PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 31/70, 37/02, 39/00
A61K 37/20

A1

(11) International Publication Number: WO 91/19502

(43) International Publication Date: 26 December 1991 (26.12.91)

(21) International Application Number: PCT/US91/04284

(22) International Filing Date: 14 June 1991 (14.06.91)

(30) Priority data:

538,853	15 June 1990 (15.06.90)	US
619,319	28 November 1990 (28.11.90)	US
632,390	21 December 1990 (21.12.90)	US
PCT/US91/03592	22 May 1991 (22.05.91)	WO

(34) Countries for which the regional or international application was filed: US et al.

(71) Applicant: CYTEL CORPORATION [US/US]; 11099 North Torrey Pines Road, Suite 100, La Jolla, CA 92037 (US).

(72) Inventors: PAULSON, James, C. ; 15255 Del Gado, Sherman Oaks, CA 91403 (US). PEREZ, Mary, S. ; 3525 Hastings Drive, Carlsbad, CA 92008 (US). GAETA, Federico, C., A. ; 8126 Camino Del Sol, La Jolla, CA 92037 (US). RATCLIFFE, Robert, Murray ; 4362 Stanford Street, Carlsbad, CA 92008 (US).

(74) Agent: SMITH, William, M.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

Published
With international search report.

(54) Title: INTERCELLULAR ADHESION MEDIATORS

(57) Abstract

The present invention relates to compositions and methods for reducing or controlling inflammation and for treating inflammatory disease processes and other pathological conditions mediated by intercellular adhesion. Compositions which selectively bind a selectin receptor and which have at least one selectin-binding moiety are provided by the present invention. The selectin-binding moieties have the general formula: R_1-Galβ1,4(Fucα1,3)GlcNAc-(R_2)a wherein R_1 is an oligosaccharide or R_3-R_4-C(CO_2H)-, wherein R_3 and R_4 are the same or different and are -H, -C1-C8 alkyl, -hydroxyl C1-C8 alkyl, -aryl C1-C8 alkyl, or -alkoxy C1-C8 alkyl; wherein R_2 is βl, 3Gal, α1, 2Man or α1, 6GalNac and a is 0 or 1.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**ES**	Spain	**MG**	Madagascar
AU	Australia	**FI**	Finland	**ML**	Mali
BB	Barbados	**FR**	France	**MN**	Mongolia
BE	Belgium	**GA**	Gabon	**MR**	Mauritania
BF	Burkina Faso	**GB**	United Kingdom	**MW**	Malawi
BG	Bulgaria	**GN**	Guinea	**NL**	Netherlands
BJ	Benin	**GR**	Greece	**NO**	Norway
BR	Brazil	**HU**	Hungary	**PL**	Poland
CA	Canada	**IT**	Italy	**RO**	Romania
CF	Central African Republic	**JP**	Japan	**SD**	Sudan
CG	Congo	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CH	Switzerland			**SN**	Senegal
CI	Côte d'Ivoire	**KR**	Republic of Korea	**SU**	Soviet Union
CM	Cameroon	**LI**	Liechtenstein	**TD**	Chad
CS	Czechoslovakia	**LK**	Sri Lanka	**TG**	Togo
DE	Germany	**LU**	Luxembourg	**US**	United States of America
DK	Denmark	**MC**	Monaco		

INTERCELLULAR ADHESION MEDIATORS

FIELD OF THE INVENTION

The present invention relates to compositions and methods for reducing or controlling inflammation and for treating inflammatory disease processes and other pathological conditions mediated by intercellular adhesion.

BACKGROUND OF THE INVENTION

Vascular endothelial cells and blood platelets play key roles in a number of biological responses by selectively binding certain cells, for instance phagocytic leukocytes, in the blood stream. For example, endothelial cells preferentially bind monocytes and granulocytes prior to their migration through the blood vessel wall and into surrounding tissue in an inflammatory response. Certain inflammation-triggering compounds are known to act directly on the vascular endothelium to promote the adhesion of leukocytes to vessel walls. Cells then move through the walls and into areas of injury or infection. Cellular adhesion to vascular endothelium is also thought to be involved in tumor metastasis. Circulating cancer cells apparently take advantage of the body's normal inflammatory mechanisms and bind to areas of blood vessel walls where the endothelium is activated.

Blood platelets are also involved in similar responses. Platelets are known to become activated during the initiation of hemostasis and undergo major morphological, biochemical, and functional changes (e.g., rapid granule exocytosis, or degranulation), in which the platelet alpha granule membrane fuses with the external plasma membrane. As a result, new cell surface proteins become expressed that confer on the activated platelet new functions, such as the ability to bind both other activated platelets and other cells. Activated platelets are recruited into growing thrombi, or are cleared rapidly from the blood circulation. Activated platelets are

known to bind to phagocytic leukocytes, including monocytes and neutrophils. Examples of pathological and other biological processes which are thought to be mediated by this process include atherosclerosis, blood clotting and inflammation.

Recent work has revealed that specialized cell surface receptors on endothelial cells and platelets, designated endothelial leukocyte adhesion molecule-1 (ELAM-1) and granule membrane protein-140 (GMP-140), respectively, are involved in the recognition of various circulating cells by the endothelium and platelets. These receptors are surface glycoproteins with a lectin-like domain, a region with homology to epidermal growth factor, and a region with homology to complement regulatory proteins (see, Bevilacqua et al., Science 243:1160 (1989), which is incorporated herein by reference). For example, ELAM-1 has been shown to mediate endothelial leukocyte adhesion, which is the first step in many inflammatory responses. Specifically, ELAM-1 binds human neutrophils, monocytes, eosinophils, certain T-lymphocytes (N. Graber et al., J. Immunol., 145:819 (1990)), NK cells, and the promyelocytic cell line HL-60.

The term "selectin" has been suggested for a general class of receptors, which includes ELAM-1 and GMP-140, because of their lectin-like domain and the selective nature of their adhesive functions. These cell surface receptors are expressed on a variety of cells. GMP-140 (also known as PADGEM) is present on the surface of platelets and endothelial cells, where it mediates platelet-leukocyte and endothelium-leukocyte interactions. Another member of the selectin class is the MEL-14 antigen, and its human analog LAM-1, which are cell surface receptors of lymphocytes, and act as lymph node homing receptors. The exact nature of the ligand recognized by selectin receptors remains unknown.

Various other methods have been previously developed to block the action of selectins and thus inhibit cellular adhesion. For instance, the use of monoclonal antibodies directed to ELAM-1 has been proposed as a method to inhibit endothelial-leukocyte adhesion as a treatment for pathological responses, such as inflammation. Endothelial interleukin-8 has

also been shown to be an inhibitor of leukocyte-endothelial interactions.

With the elucidation of the ligand-receptor interaction, it will be possible to develop highly specific, efficient inhibitors of selectin-mediated cellular adhesion which would be useful in therapeutic regimens. The ligand(s) could also be used to target other pharmaceutical compounds, such as anti-inflammatory agents or anti-oxidants, to the sites of injury. To date, insufficient understanding of the interaction of the ligand(s) and receptor molecules on the respective cells has hindered these efforts. The present invention fulfills these and other related needs.

SUMMARY OF THE INVENTION

Novel compositions which selectively bind a selectin receptor and which have at least one selectin-binding moiety are provided by the present invention. The selectin-binding moieties have the general formula:

R_1–Galβ1,4(Fucα1,3)GlcNAc–$(R_2)_a$

wherein R_1 is an oligosaccharide or R_3–R_4–C(CO_2H)–,

wherein R_3 and R_4 are the same or different and are –H, – C1-C8 alkyl, – hydroxyl C1-C8 alkyl, –aryl C1-C8 alkyl, or –alkoxy C1-C8 alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

In certain preferred embodiments, R_1 is a sialic acid, usually NeuAc or NeuGc. If R_1 is an oligosaccharide it will preferably be NeuAcα2,3Galβ1,4GlcNAcβ1,3 or NeuGcα2,3Galβ1,4GlcNAcβ1,3. The selectin binding moieties are typically oligosaccharides comprising the minimal tetrasaccharide (termed SLX) recognized by the selectin receptor.

The compound is typically prepared from a polysaccharide having a repeat unit comprising the unfucosylated SLX core structure. Upon fucosylation, a polyvalent SLX-bearing polysaccharide is obtained. Preferred polysaccharides for this purpose are type Ia, type II, and type III, polysaccharides from Group B streptoccus. Polyvalent

selectin-binding compounds are also obtained by linking selectin-binding moieties to various linker moieties.

The claimed compositions inhibit intercellular adhesion mediated by the selectin cell surface receptor and thereby are capable, for example, of inhibiting inflammatory and other pathological responses associated with cellular adhesion. In related embodiments the composition that binds the selectin may be a polysaccharide, a glycoprotein, a glycolipid, or an oligosaccharide.

The present invention specifically provides the above compounds in pharmaceutical compositions. The pharmaceutical compositions can comprise, for example, liposomes which comprise a moiety capable of selectively binding a selectin receptor and a pharmaceutically acceptable carrier. The liposome having the moiety may also serve as a targeting vehicle for a conventional chemotherapeutic agent, which agent is encapsulated within the liposome and delivered to targeted cells which express a selectin receptor. Typically the chemotherapeutic agent is an anti-inflammatory agent or an anti-oxidant. Using the moieties described herein to target chemical agents encapsulated within liposomes is a convenient and effective method for reducing therapeutic levels of a drug and minimizing side effects.

The pharmaceutical compositions of the present invention may also comprise immunoglobulins capable of selectively binding an oligosaccharide ligand recognized by a selectin receptor. Suitable immunoglobulins for this purpose include CSLEX-1, FH6, SNH_3, SNH_4 and VIM-2.

In other aspects, the invention comprises methods of inhibiting intercellular adhesion in a patient for a disease process, such as inflammation, by administering to the patient a therapeutically effective dose of a compound comprising a moiety capable of binding a selectin receptor. The selectin receptor, such as ELAM-1 or GMP-140, may be expressed on vascular endothelial cells or platelets. The inflammatory process may be, for example, septic shock, wound associated sepsis, acute respiratory

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the ability of cells which express SLX (LEC 11) to bind to IL-1β activated endothelial cells compared to those cells which express non-sialylated Le^x (CHO-K1 and LEC 12).

Fig. 2 illustrates the ability of monoclonal antibodies specific for SLX to block selectin-mediated binding of HL-60 cells at 37°C (Fig. 2A) and 4°C (Fig. 2B) compared to monoclonal antibodies which do not bind SLX determinants.

Fig. 3 illustrates the effects of incubating LEC 11 (Fig. 3A) and LEC 12 (Fig. 3B) cells with SLX and non-SLX specific monoclonal antibodies on binding to activated endothelial cells.

Fig. 4 illustrates the results obtained by treating HL-60, LEC11 and LEC12 cells with sialidase before binding to activated endothelial cells.

Fig. 5 compares the ability of liposomes which contain glycolipids with SLX, Le^x, or similar carbohydrate structures to inhibit the binding of HL-60 cells to activated endothelial cells.

Fig. 6 compares the inhibition of GMP-140 mediated platelet adhesion by monoclonal antibodies specific for SLX and Le^x determinants.

Fig. 7 compares the ability of liposomes which contain glycolipids with SLX, Le^X, or similar carbohydrate structures to inhibit the binding of HL-60 cells to activated platelets.

Fig. 8 compares the ability of liposomes which contain glycolipids with SLX, Le^X, or similar carbohydrate structures to inhibit the binding of PMNs to activated platelets.

Fig. 9 shows inhibition of GMP-140 mediated adhesion by glycolipids with the terminal sialic acid either NeuAc or NeuGc.

Fig. 10 shows prophylactically administered monoclonal antibodies against ELAM-1 prevent lipopolysaccharide induced death in rate.

Fig. 11 shows therapeutically administered monoclonal antibodies against ELAM-1 prevent lipopolysaccharide induced death in rats.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Compositions and methods are provided for inhibiting inflammatory and other disease responses mediated by cellular adhesion. The present invention also provides compounds (e.g., glycoconjugates and monoclonal antibodies) which have the ability to block or inhibit the adhesion of the cells mediated by selectin cell surface receptors. Methods for preparing and screening for such compounds are also provided. Diagnostic and therapeutic uses for the compounds are provided.

A basis of the present invention is the discovery of a carbohydrate moiety recognized by selectin cell surface receptors. As discussed above, selectins, also known as the "LEC-CAM" family of cell adhesion molecules, are unique glycoproteins expressed on the surface of a variety of cells. For instance, ELAM-1 is inducibly expressed on vascular endothelial cells (Bevilacqua et al., supra and Hession et al., Proc. Nat'l. Acad. Sci., 87:1673-1677 (1990), both of which are incorporated herein by reference). This receptor has been demonstrated to be induced by inflammatory cytokines such as interleukin Iβ (IL-Iβ) and tumor necrosis factor α (TNFα), as well as bacterial endotoxin (lipopolysaccharide) (see, Bevilacqua et al., Proc. Natl. Acad. Sci., 84:9238-9242 (1987) which is incorporated herein by reference). These compounds act directly on endothelial cells in vitro to substantially augment polymorphonuclear leukocyte (neutrophil), and monocyte adhesion (Bevilacqua et al., Proc. Natl. Acad. Sci., supra).

As discussed above, GMP-140 is a membrane glycoprotein of platelet and endothelial secretory granules (Geng et al., Nature, 343, 757-760 (1990) which is incorporated herein by reference). Activated platelets which express GMP-140 on their surface are known to bind to monocytes and neutrophils (Jungi et al., Blood 67:629-636 (1986)), and also to monocyte-like cell lines, e.g., HL60 and U937 (Jungi et al., supra; Silverstein et al., J. Clin. Invest. 79:867-874 (1987)),

all of which are incorporated herein by reference. GMP-140 is an alpha granule membrane protein of molecular weight 140,000 that is expressed on the surface of activated platelets upon platelet stimulation and granule secretion (Hsu-Lin et al., J. Biol. Chem. 259:9121-9126 (1984); Stenberg et al., J. Cell Biol. 101:880-886 (1985); Berman et al., J. Clin. Invest. 78:130-137 (1986)). It is also found in megakaryocytes (Beckstead et al., Blood 67:285-293 (1986)), and in endothelial cells (McEver et al., Blood 70:355a (1987)) within the Weibel-Palade bodies (Bonfanti et al., Blood 73:1109-1112 (1989)). Furie et al. U.S. Patent No. 4,783,330, describe monoclonal antibodies reactive with GPM-140. All of the foregoing references are incorporated herein by reference.

A third selectin receptor is the lymphocyte homing receptor, MEL-14 antigen or LAM-1 (Gallatin et al., Nature 304:30-34 (1983); Siegellman et al., Science, 243:1165-1172 (1989); Rosen, Cell Biology, 1:913-919 (1989); and Lasky et al. Cell 56:1045-1055 (1989) all of which are incorporated herein by reference). In addition to lymphocyte homing, MEL-14 antigen/LAM-1 is believed to function early in neutrophil binding to the endothelium.

The structure and function of selectin receptors has been elucidated by cloning and expression of full length cDNA encoding each of the above receptors (see, e.g., Bevilacqua et al., Science, supra, (ELAM-1), Geng et al., supra, (GMP 140), and Lasky et al., supra, (MEL-14 antigen)). The extracellular portion of selectins can be divided into three segments based on homologies to previously described proteins. The N-terminal region (about 120 amino acids) is related to the C-type mammalian lectin protein family as described by Drickamer, J. Biol. Chem., 263: 9557-9560 (1988) (which is incorporated herein by reference) that includes low affinity IgE receptor CD23. A polypeptide segment follows, which has a sequence that is related to proteins containing the epidermal growth factor (EGF) motif. Lastly, after the EGF domain are one or more tandem repetitive motifs of about 60 amino acids each, related to those found in a family of complement regulatory proteins.

Since selectin receptors comprise a lectin-like domain, the specificity of the molecules is likely to be based on protein-carbohydrate interactions. Evidence provided here indicates that a sialylated, fucosylated N-acetyllactosamine unit of the Lewis X antigen, designated here as SLX, is a moiety recognized by the lectin region of the selectin receptor. In particular, the evidence shows recognition of this moiety by both ELAM-1 and GMP-140. Compounds of the present invention comprise this fucosylated, sialylated N-acetyllactosamine unit in a variety of configurations.

Selective binding as used herein refers to specific recognition by one molecule (typically referred to as a receptor) of another molecule (typically referred to as a ligand) by the spatial or polar organization of a determinant site on the second molecule. Selective binding between the two molecules occurs where affinity is sufficiently strong. Binding affinity is typically represented by the affinity constant (K_a) for equilibrium concentrations of associated and disassociated configurations, i.e., K_a = [R-L]/[R][L] where [R], [L], and [R-L] are the concentrations at equilibrium of the receptor (R), ligand (L) and receptor-ligand complex (R-L), respectively.

The specific binding interactions of the receptor and ligand molecules typically include reversible noncovalent associations such as electrostatic attraction, Van der Waals forces, and hydrogen bonds. See generally, Stryer, Biochemistry (W.H. Freeman and Company, N.Y. 3rd Ed. 1988), which is incorporated herein by reference. Examples of selective binding interactions include antibody-antigen recognition, enzyme-substrate recognition, and the like.

The nomenclature used to describe the oligosaccharide moieties of the present invention follows the conventional nomenclature. Standard abbreviations for individual monosaccharides are used. For instance, 2-N-acetylglucosamine is represented by GlcNAc, fucose is Fuc, galactose is Gal, and glucose is Glc. Two sialic acids which may be present on the oligosaccharides of the present invention are 5-N-acetylneuraminic acid (NeuAc) and 5-N-glycolylneuraminic acid

(NeuGc). Unless otherwise indicated, all sugars except fucose (L-isomer) are D-isomers in the cyclic configuration (e.g., pyranose or furanose). The two anomers of the cyclic forms are represented by α and β.

The monosaccharides are generally linked by glycosidic bonds to form oligo- and polysaccharides. The orientation of the bond with respect to the plane of the rings is indicated by α and β. The particular carbon atoms that form the bond between the two monosaccharides are also noted. Thus, a β glycosidic bond between C-1 of galactose and C-4 of glucose is represented by Galβ1,4Glc. For the D-sugars (e.g., D-GlcNAc, D-Gal, and D-NeuAc) the designation α means the hydroxyl attached to C-1 (C-2 in NeuAc) is below the plane of the ring and β is above the ring. In the case of L-fucose, the α designation means the hydroxyl is above the ring and β means it is below.

Having identified SLX as a carbohydrate ligand that mediates leukocyte-endothelial and leukocyte-platelet cell adhesion, compounds comprising SLX and related structures can be purified or synthesized de novo. As detailed below, the present invention provides a variety of compounds comprising the selectin-binding moieties of the present invention. For instance, biomolecules can be used as the moiety-bearing compound. Biomolecules as defined here include but are not limited to biologically significant molecules such as amino acids (and their mimetics), oligopeptides, proteins (e.g., glycoproteins and protein hormones), fatty acids, lipids (e.g., glycolipids, phospholipids, sphingolipids and gangliosides), steroid hormones, oligosaccharides, polysaccharides, and nucleic acids (e.g., deoxyribonucleic acids and ribonucleic acids). These compounds can be purified and/or synthesized according to standard techniques known to the skilled artisan. In addition, a wide variety of compounds bearing the moiety may be synthesized de novo as described below.

Once obtained, such compounds can be used for a variety of purposes, including, for example, competitive inhibition of the binding of SLX-bearing cells to cells which express the selectin receptors. By binding of the compounds of

the invention to a cell surface selectin, interaction of the selectin with the native SLX ligand on migrating cells will be prevented, interfering with normal and pathological binding of leukocytes and other cells to the endothelium or platelets. Thus, compounds which contain one or more selectin-binding moieties can serve as effective inhibitors of, for instance, inflammation, atherosclerosis, clotting and other endothelial or platelet-mediated pathologies.

The sialic acid residue in SLX may be in different forms, so long as selectin binding is not significantly affected. Typically, the sialic acid is 5-N-acetylneuraminic acid, (NeuAc) or 5-N-glcolylneuraminic acid (NeuGc). Other sialic acids may be used in their place, however. For a review of different forms of sialic acid suitable in the present invention see generally, R. Schauer, Methods in Enzymology, 50: 64-89 (1987), and Schaur, Advances in Carbohydrate Chemistry and Biochemistry, 40: 131-234; both of which are incorporated by reference. As demonstrated in Example IX, below, the affinity for selectin receptors is the same if the oligosaccharide terminates in NeuAc or NeuGc. Thus, the term "SLX" as used herein refers to the minimal tetrasaccharide unit (sialic acidα2,3Galβ1,4[Fucα1,3]GlcNA(β1,3) in which the sialic acid is NeuAc, NeuGc or other equivalent forms of sialic acid. Structures illustrated herein which show the sialic acid residue as NeuAc are understood to include these other forms, in particular NeuGc.

Evidence provided below shows that a pentasaccharide comprising the formula:
NeuAcα2,3Galβ1,4[Fucα1,3]GlcNAcβ1,3Galβ– is a minimal structure having substantially more inhibitory effect than the tetrasaccharide. Thus, in certain preferred embodiments the oligosaccharide will comprise this pentasaccharide structure.

Other variations on the basic SLX unit are also recognized by selectin receptors. For instance, evidence provided in Example VIII, below, shows that an oligosaccharide moiety, termed SY2 (also known as the VIM-2 antigen), having the structure
NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ

binds selectin receptors equally as well as SLX. The SY2 moiety comprises two sialylated N-acetyllactosamine units, one of which is SLX. Thus, oligosaccharides recognized by selectin receptors may comprise a number of the sialylated N-acetyllactosamine units, at least one of which is fucosylated (see, Teimeyer et al., Proc. Natl. Acad. Sci. (USA) 88:1138-1142 (1991), which is incorporated herein by reference.

The oligosaccharide moiety of the present invention preferably terminates in a sialic acid residue. In certain embodiments the sialic acid residue can be further linked to other saccharide residues, such as a second sialic acid in an α2,8 linkage.

Alternatively, the terminal sialic acid residue may be replaced by a variety of radicals. Thus, certain selectin binding moieties of the present invention have the general formula: R_1-NeuAcα2,3Galβ1,4GlcNAcβ1-,
wherein R_1 is $R_2R_3C(CO_2H)$-,
wherein R_2 and R_3 are the same or different and are H, lower alkyl (C1-C8), hydroxyl lower alkyl (C1-C8), arylalkyl, alkoxylalkyl. In addition, R_2 and R_3 may be connected to form a 4-8 membered carbocyclic or heterocyclic ring.

Compounds containing SLX and related structures can be obtained from the cell surface glycoproteins or glycolipids from a number of cells. For instance, the SLX antigen is present on N-linked carbohydrate groups of the cell surface glycoproteins of LEC11 cells, a glycosylation mutant of CHO cells. LEC11 expresses this unique glycopeptide which contains a terminal structure bearing both sialic acid and fucose in the SLX sequence:

NeuAcα2,3Galβ1,4GlcNAcβ1-R
|α1,3
Fuc

where R is:

2Man Fuc
|α1,6 ±|α1,6
Manβ1,4GlcNAcβ1,4GlcNAcβ1,Asn
|α1,4
(±SLX)β1,2Man

(See, Stanley et al., J. Biol. Chem., 263:11374 (1988), which is incorporated herein by reference.) Using the procedure described below, it was demonstrated that the LEC11 mutant bound to activated human vascular endothelial cells. Neither wild type CHO cells nor other related glycosylation mutant CHO cell lines without the particular glycosylation pattern (SLX) showed the same level of binding.

Other sources that can be used to obtain the SLX unit include any cell which naturally expresses the moiety on glycolipid or glycoprotein carbohydrate groups. Thus, polymorphonuclear neutrophils, lymphocytes, tumor cells or HL-60 cells have been used to purify this unit. Other cells which bind to activated vascular endothelium can also be used to isolate the ligand (see, Symington et al., J. Immunol. 134:2498-2506 (1985), Mizoguchi et al., J. Biol. Chem. 259:11949-11957 (1984), Mizoguchi et al., J. Biol. Chem. 259:11943-11948 (1984), Paietta et al., Cancer Res. 48:28-287 (1988), all of which are incorporated herein by reference).

Compounds containing SLX or its mimetics can be prepared from natural sources using methods well known in the art for isolating surface glycoproteins, glycopeptides, oligosaccharides and glycolipids from cells (See, e.g., Gerard, "Purification of glycoproteins" and Thomas et al., "Purification of membrane proteins," both in Guide to Protein Purification, Vol. 182, Methods in Enzymology (Deutscher ed., 1990), which is incorporated herein by reference). For example, LEC11 cells can be used to obtain glycoprotein or glycolipid which contains the SLX unit using, for instance, the method described in Stanley et al., supra. Briefly, LEC11 cells are infected with vesicular stomatitis virus. The structural carbohydrate alterations exhibited by LEC11 are then expressed on the N-linked biantennary carbohydrates of the G glycoprotein of the virus. The virus is purified by equilibrium gradient centrifugation, and glycopeptides are purified using proteinase digestion as described by Stanley et al.

Several approaches are used to isolate a selectin-binding moiety from HL-60, HT-29, colo 205, neutrophils, and

other cell lines which contain a ligand recognized by selectins. Since the ligand is generally expressed on the cell surface of these cell types, one approach consists of isolating a plasma membrane fraction enriched in the ligand. Once plasma membranes have been isolated, the ligands may be isolated and subsequently identified using monoclonal antibodies, particularly those which are reactive with the SLX oligosaccharide and related structures, such as monoclonal antibodies FH6, SNH3 and CSLEX-1.

To characterize a selectin ligand on a glycoprotein, release of the oligosaccharide is generally the first step in the structural analysis of the oligosaccharide chain. This is accomplished by chemical cleavage of the protein-carbohydrate linkage, or by specifically releasing the oligosaccharide with endoglycosidases. In most cases, different procedures may be used to establish the correct conditions for an individual glycoprotein. Asparagine-linked oligosaccharides are released by hydrazinolysis, endoglycosidases, vigorous alkaline hydrolysis, and trifluoroacetolysis. O-linked carbohydrate units are released by alkaline β-elimination. The oligosaccharides are separated from the glycopeptides by gel filtration. The resulting oligosaccharides are then separated from each other using a combination of gel filtration, HPLC, thin layer chromatography, and ion exchange chromatography. The isolated oligosaccharides are then fully analyzed. Complete structural analysis of the purified oligosaccharide units requires the determination of the monosaccharide units, their ring form, configuration (D or L), anomeric linkage (α or β), the positions of the linkages between the sugars and their sequence. In addition, the position of any substituent groups are established. Methylation analysis is used to determine the positions of the glycosidic linkages between the monosaccharides. The anomeric configuration of the sugar residues can be addressed using 500-MHz ^{1}H NMR spectroscopy. The conditions and methods used to perform a complete structural carbohydrate analysis are described generally in Beeley, Laboratory Techniques in Biochemistry and Molecular

Biology, eds. Burdon and Knippenberg, Elsevier, Amsterdam (1985), incorporated herein by reference.

The state of the art techniques to fully characterize the sugars of an oligosaccharide include the use of several analytical techniques such as FAB-MS (fast atom bombardment-mass spectrometry), HPAE (high pH anion exchange chromatography) and ^{1}H-NMR. These techniques are complementary. Recent examples of how these techniques are used to fully characterize the structure of an oligosaccharide can be found in the analysis by Spellman et al., J. Biol. Chem. 264:14100 (1989), and Stanley et al., supra. Other methods include positive ion fast atom bombardment mass spectroscopy (FAB-MS) and methylation analysis by gas chromatography - electron impact mass spectroscopy (GC/EI-MS) (see, EPO Application No. 89305153.2, which is incorporated herein by reference).

One approach to characterizing the selectin ligand on glycolipids consists of disrupting the cells using organic solvents, isolating the glycolipids, and identifying those glycolipids reactive with monoclonal antibodies to SLX, such as FH6, SNH3, SNH4, CSLEX-1, or VIM-2, for example, and then determining the structure of the oligosaccharide chains. To obtain glycolipids and gangliosides which contain SLX, standard methods for glycolipid preparation can be used (see, e.g., Ledeen et al., J. Neurochem. 21:829 (1973), which is incorporated herein by reference). For example, glycolipids are extracted from HL-60, HT-29, PMNs, human leukocytes, and other cell lines expressing the selectin ligand by methods generally known to those skilled in the arts (see, e.g., Symington et al., J. Immunol. 134:2498 (1985) and Macher and Beckstead, Leukemia Res. 14:119-130 (1990), which are incorporated herein by reference). Cells are grown in suspension and are harvested by centrifugation. Glycolipids are extracted from the cell pellet by chloroform/methanol 2:1 and isopropyl alcohol/hexane/water 55:25:20 as described by Kannagi et al., J. Biol. Chem. 257:14865 (1982), which is incorporated herein by reference. The resulting extracts are partitioned by a chloroform/methanol/water (3:2:1) Folch

partition. The resulting upper phase of the extraction contains gangliosides and the lower phase contains glycolipids.

The upper phase containing gangliosides (glycosphingolipids that contain at least one sialic acid moiety) are isolated and separated into neutral and acidic fractions using DEAE-Sephadex chromatography as described in detail by Ledeen and Yu, Methods Enzymol. 83:139 (1982), which is incorporated herein by reference. The resulting gangliosides are pooled, lyophilized, and dissolved in chloroform/methanol (2:1). The lower phase of the Folch partition contains glycolipids. These are isolated and separated on preparative thin-layer chromatography using chloroform/methanol/water (60:35:8) as the solvent system as described by Symington.

To identify those gangliosides and glycolipids which contain a selectin ligand, immunochemical glycolipid analysis is performed according to the procedure of Magnani et al., Anal. Biochem. 109:399 (1980), which is incorporated herein by reference. Briefly, the ganglioside pool described above is chromatographed by thin layer chromatography. The thin layer plate is then incubated with ^{125}I labeled CSLEX-1, or other monoclonal antibody which binds specifically to SLX or related structures. Following incubation with the labeled antibody, the plate is exposed to radiographic detection film and developed. Black spots on the X-ray film correspond to gangliosides that bind to the monoclonal antibody, and those gangliosides are recovered by scraping the corresponding areas of the silica plate and eluting the gangliosides with chloroform/methanol/water. Glycolipids are also dried and resuspended in chloroform and developed in a similar thin layer system and probed with the radiolabeled antibody. Structural analysis of oligosaccharides derived from glycolipids is performed essentially as described for glycoproteins.

Oligosaccharides comprising the SLX unit can be prepared from glycoproteins by methods well known in the art (see, e.g., Gerard, supra, at pp. 537-539). Typically, N-glycosidase F (N-glycanase) is used to cleave N-linked

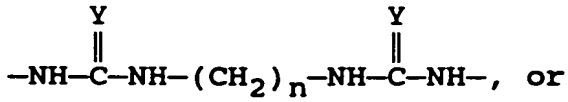
oligosaccharides while O-linked groups are cleaved with endo-N-acetylgalactosaminidase.

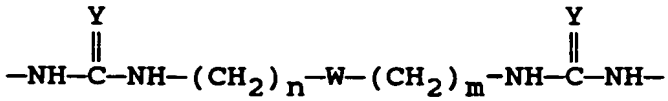
Synthetic compounds containing SLX or its mimetics attached to a variety of moieties can be prepared depending on the particular use desired. For example, SLX can be converted to a ganglioside by linking a ceramide moiety to the C-1 of the reducing terminal GlcNAc unit. SLX structures and related structures can also be linked to a wide variety of other moieties such as variously substituted amino groups, heterocyclic compounds, ether linkages with branched or unbranched carbon chains, and ether linkages with aryl or alkylaryl moieties. The selectin-binding moiety may also be bound to various polysaccharides, amino acids, amino acid mimetics, oligopeptides or proteins using methods well known in the art.

The term "alkyl" as used herein means a branched or unbranched saturated or unsaturated hydrocarbon chain, including lower alkyls of 1-8 carbons such as methyl, ethyl, n-propyl, butyl, n-hexyl, and the like, cycloalkyls (3-7 carbons), cycloalkylmethyls (4-8 carbons), and arylalkyls. The term "aryl" refers to a radical derived from an aromatic hydrocarbon by the removal of one atom, e.g., phenyl from benzene. The aromatic hydrocarbon may have more than one unsaturated carbon ring, e.g., naphthyl. The term "alkoxyl" refers to alkyl radicals attached to the remainder of the molecule by an oxygen, e.g., ethoxyl. The term "heterocyclic compounds" refers to ring compounds having three or more atoms in which at least one of the atoms is other than carbon (e.g., N, O, S, Se, P, or As). Examples of such compounds include furans, pyrimidines, purines, pyrazines and the like. The term "oligo" refers to a polymeric molecule consisting of 2 to approximately 10 residues, for example, amino acids (oligopeptide), monosaccharides(oligosaccharide), and nucleic acids (oligonucleotide). The term "poly" refers to a polymeric molecule comprising greater than 10 residues.

For the synthesis of polyvalent forms of selectin-binding moieties, monomeric units containing SLX or other structures can be joined to form molecules having one to about

four or more selectin-binding moieties. An example of such a polyvalent form is one in which the units are linked by the following linking moieties:

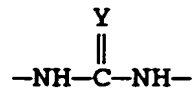




wherein, n and m are the same or different and are integers from 2 to 12; Y is O or S; and W is O, S, or NH.

Alternatively,

the moiety is a 5- to 14-membered ring having two substituents, each substituent having the formula



wherein, Y is O or S, the substituents being in a cis- or trans-relationship.

SLX and related structures may also be attached to various a heterocyclic compounds (e.g., one comprising nitrogen atoms). In this case, the moieties are preferably linked to the nitrogen atoms on the ring, each nitrogen being linked to one moiety. Examples of heterocyclic compounds that are suitable for this purpose include piperazine and homopiperazine.

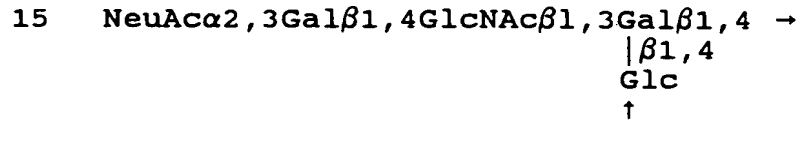
Alternatively, polyvalent forms of SLX or its mimetics can be created by attaching the desired moiety to preformed carrier moieties with multiple sites of attachment. Examples include attachment of SLX to the amino groups of lysine and lysine-containing peptides, proteins, glycoproteins or the asparagine side-chain of such compounds.

Another method of preparing polyvalent selectin-binding compounds is by addition of desired monosaccharide residues to polysaccharides. For instance, a polysaccharide which contains a repeat unit having the linear core structure of SLX (i.e, without the fucose residue) may be converted into a polyvalent SLX containing polysaccharide by enzymatic

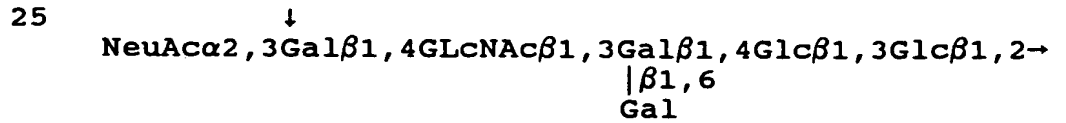
fucosylation. Native polysaccharide types Ia, II, or III obtained from Group B Streptococcus are preferably used for this purpose. These polysaccharides can be isolated according to standard procedures from cell lines deposited with the American Type Culture Collection (Type Ia from ATCC Nos. 12400 and 31574; Type II from ATCC Nos. 12973 and 31576; and Type III from ATCC No. 31577). See e.g., Jennings et al., Biochem. 22 1258-1263 (1983) and PCT Application, Publication No. 8706267, both of which are incorporated herein by reference.

These polysaccharides comprises repeat units having the formulas:

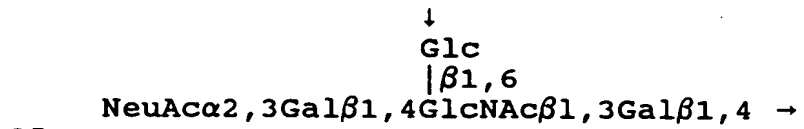
Type Ia



Type II

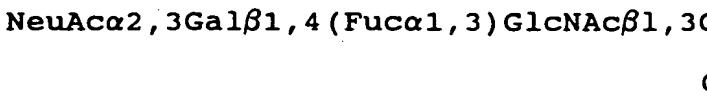


Type III



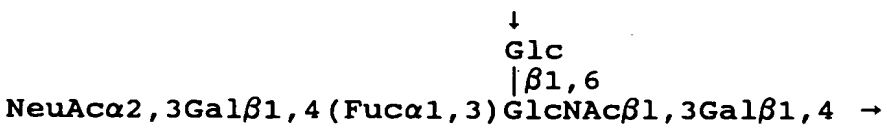
The arrows in the above structures identify the backbone in each polysaccharide molecule. As can be seen, type Ia polysaccharides contain a repeat unit having side chain which corresponds to the SLX linear core structure. The other two polysaccharides, the backbone comprises the SLX core structure. Enzymatic fucosylation of these polysaccharides using an α1,3 fucosyltransferase according to standard techniques described below yields a polyvalent SLX compound. After fucosylation, the repeat units have the following formulas:

Type Ia



Type II

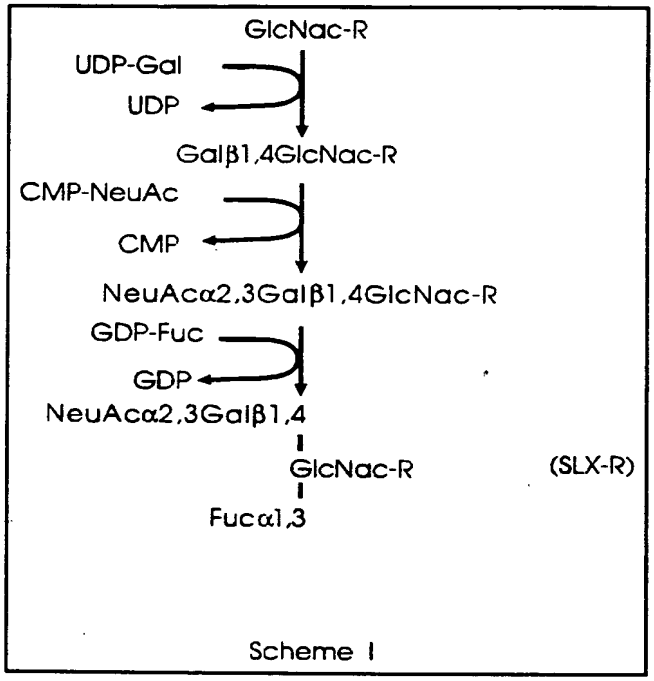
Type III



The entire polysaccharide can be used for this purpose as well as fragments thereof. Thus, polysaccharides having a molecular weight between about 5,000 and about 300,000 can be used. A molecular weight between about 25,000 and about 100,000 is preferred. Any number of side chains on the polysaccharide type Ia may be fucosylated for the polysaccharide to have activity. Typically, between about 5 and about 200 side chains are fucosylated, preferably between about 50 and about 150 are fucosylated.

The synthesis of the selectin-binding moiety can be accomplished using chemical, enzymatic, or combined chemical and enzymatic strategies. (see, e.g., EPO Publication No. 319,253, which is incorporated herein by reference.) In a preferred method (Scheme I below), a compound containing one or more N-acetylglucosamine units (GlcNAc-R) can be reacted sequentially with a galactosyltransferase (N-acetylglucosamine β1,4 galactosyltransferase (E.C. 2.4.1.90)), a sialytransferase (Galβ1,4GlcNAc α2,3 sialyltransferase (E.C. 2.4.99.6) or Galβ1,3GalNAc α2,3 sialytransferase (E.C. 2.4.99.4) and a fucosyltransferase (N-acetylglucosaminide α1,3 fucosyltransferase (E.C. 2.4.1.152)) to yield the final SLX-containing structures. In this case, R may be a carrier moiety

or activatable intermediate that will allow attachment to a suitable carrier moiety. Each enzymatic reaction uses the appropriate nucleotide sugar as a donor substrate to generate the following intermediates in the synthesis of SLX. The glycosyl transfer reactions may optimally be carried out with added alkaline phosphatase (e.g., from calf intestine, CIAP) to consume the nucleoside phosphate byproduct which may inhibit the reaction.



Scheme I

The general conditions for preparative enzymatic synthesis of carbohydrate groups analogous to SLX are known (see, e.g., Toone et al., Tetrahedron 45:5365-5422 (1989); Wong et al., J. Am. Chem. Soc. 47:5416-5418 (1982); Unverzagt et al., J. Am. Chem. Soc. 112:9308-9309 (1990); Prieels et al., J. Biol. Chem. 256:10456-10463 (1981), all of which are incorporated herein by reference). Each of the key enzymatic reactions has been demonstrated (Beyer et al., Adv. Enzymol. 52:23-176 (1981); Toone et al., supra; and Howard et al., J. Biol. Chem. 262:16830-16837 (1981); all of which are incorporated herein by reference). For preparative reactions, the galactosyltransferase and the sialyltransferase(s) are purified from natural sources (Beyer et al., supra, and Weinstein et al., J. Biol. Chem. 257:13835-13844 (1982), which

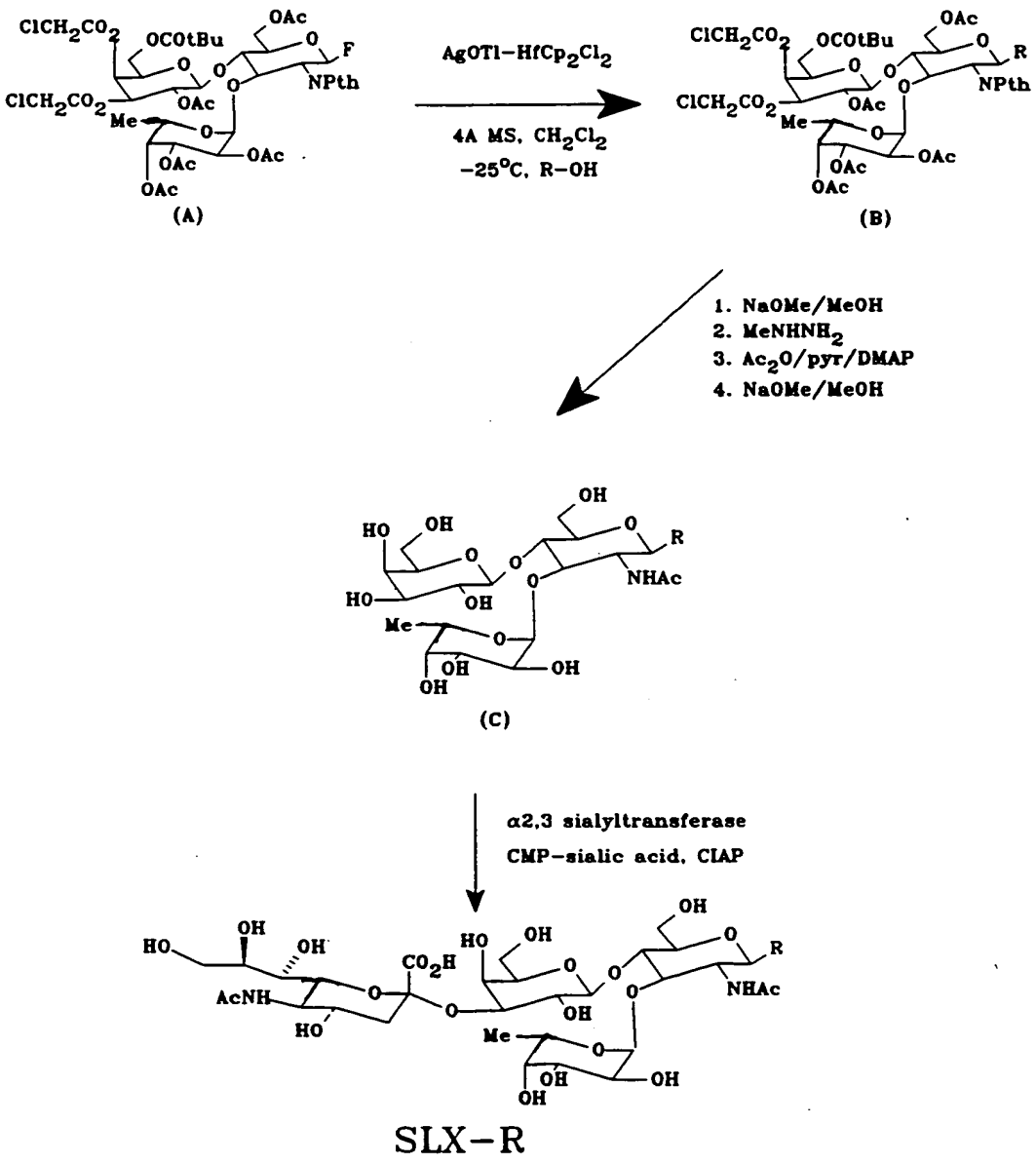
are incorporated herein by reference). Fucosyltransferases may also be identified from natural sources, as generally described in Crawley and Hindsgaul, Carbohyd. Res. 193:249-256 (1989), incorporated by reference herein. The cDNAs of the galactosyltransferase and a sialyltransferase have been cloned (Paulson and Colley, J. Biol.Chem. 264:17615-17618 (1989), which is incorporated herein by reference), allowing the production of soluble recombinant enzymes for large-scale preparative synthesis (Colley et al., J. Biol. Chem. 264:17619-17622 (1989)).

To obtain sufficient amounts of fucosyltransferase for large-scale reaction, the enzyme can be cloned and expressed as a recombinant soluble enzyme by someone with ordinary skill in the art. As a preferred method RNA can be subtracted from the wild type CHO cells and LEC11 cells as described by Chirgwin et al., Biochemistry 18:5214-5299 (1979), and the poly A+ RNA isolated by chromatography on oligo(dT)-cellulose. Next, cDNA from the LEC-11 cells can be prepared as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor Press, New York, which is incorporated herein by reference. The cDNA can be subtracted using the method of Davis (Handbook of Experimental Immunology, Vol. 2, pp. 1-13 (1986)) using excess poly A+ RNA from wild type CHO cells, which do not express the desired fucosyltransferase, but otherwise have most of the mRNA species of LEC11 cells. A cDNA library can then be constructed in the CDM8 expression vector using the subtracted cDNA (Seed, Nature 329:840-842 (1987)). Clones expressing the fucosyltransferase can be isolated using the expression cloning method described by Larsen et al., Proc. Natl. Acad. Sci. 86:8227-8231 (1989), employing transfection of COS-1 cells and screening for cells expressing the SLX antigen with the CSLEX antibody or other antibody with specificity for the SLX antigen. The full-length clone of the fucosyltransferase can then be used to produce a soluble recombinant enzyme as taught by Colley et al., supra.

Another source of SLX is α_1-acid glycoprotein, which is a plasma glycoprotein, the carbhydrate moieties of which can

be fucosylated to produce SLX (see, Alpha,-Acid glycoprotein: Genetics, Biochemistry, Physiological Functions, and Pharmacology, Bauman et al. ed. (Wiley 1989), and Walz, et al. Science 250:1132-1135 (1990), both of which are incorporated herein by reference).

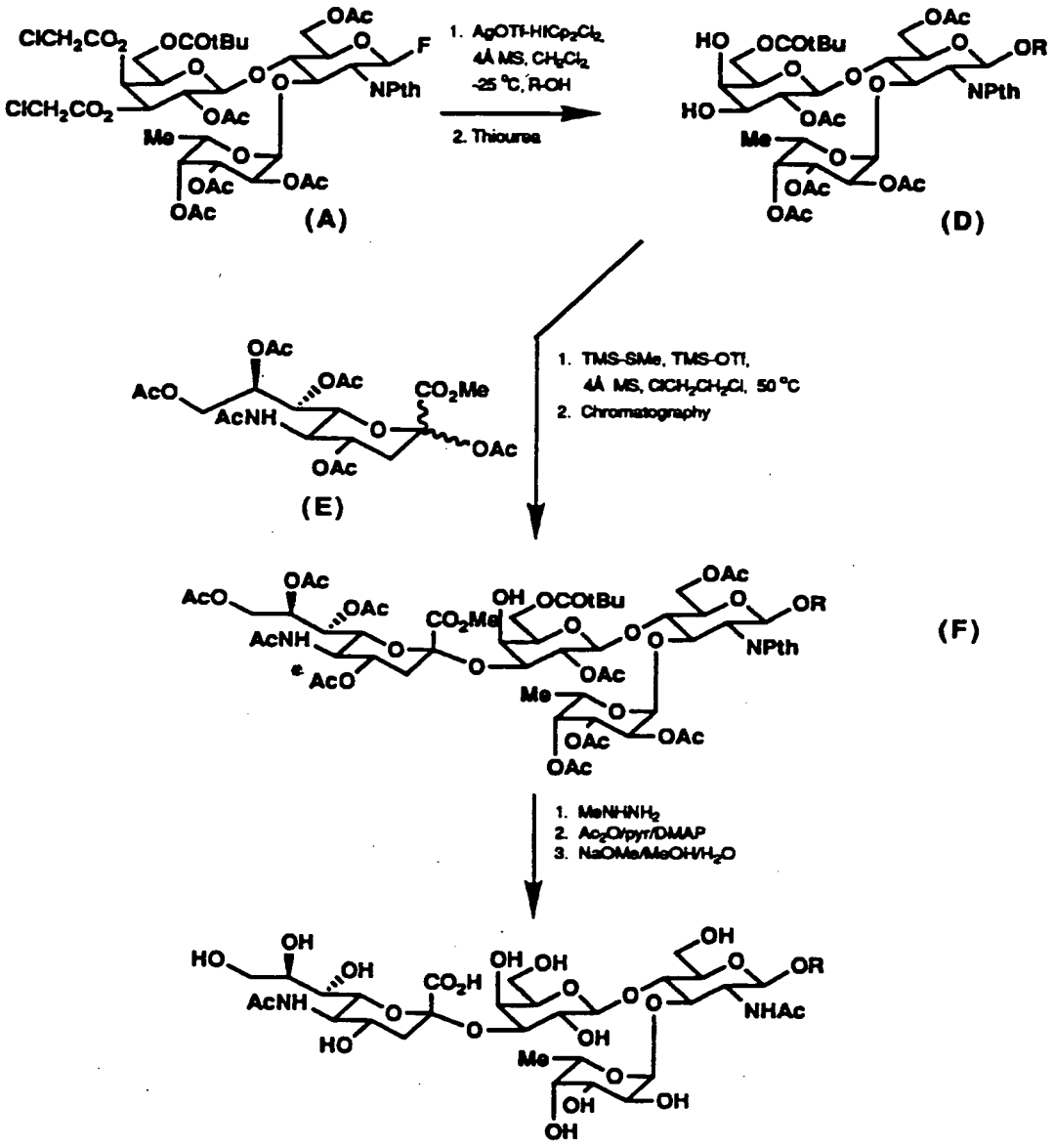
Although enzymatic or combined chemical and enzymatic synthesis of SLX compounds are preferred, chemical synthesis is also possible, as shown in Schemes II and IIa below.



(A) (B) (C)

SLX–R

Scheme II


1. AgOTf-HfCp2Cl2, 4Å MS, CH2Cl2, -25 °C, R-OH
2. Thiourea
(A)
(D)
1. TMS-SMe, TMS-OTf, 4Å MS, ClCH2CH2Cl, 50 °C
2. Chromatography
(E)
(F)
1. MeNHNH2
2. Ac2O/pyr/DMAP
3. NaOMe/MeOH/H2O
SLX-R

Scheme IIa

Component pieces of the SLX structure have been synthesized. For instance, the preparation of sialic acid containing glycosides, including SLX, is disclosed in European Patent Application No. 88311312.8, which is incorporated herein by reference. Nicolaou, et al., (J. Amer. Chem. Soc. 112:3693 (1990)) have published the total synthesis of the tumor-associated Le^x family of glycosphingolipids. Therein is described the synthesis of the protected trisaccharide Galβ1,4(Fucα1,3)GlcNAc (A) as illustrated in Scheme II. Reaction of this intermediate with an appropriate glycosyl acceptor (e.g., an alcohol moiety) results in compound (B). Selective deprotection and acetylation of the glucosamine moiety are carried out essentially as described in Nicolaou, et al. to afford compound (C). Reaction of (C) with a sialyltransferase as described above furnishes the desired product SLX-R, although this may be produced in relatively low yield using Scheme II.

Modified fucosides may be included in the synthetic schemes to provide for SLX analogues which vary in this moiety. For example, α-D-arabinosyl glycosides may be synthesized following known procedures, Nicolaou et al., J.Amer.Chem.Soc. 112:3693-3695 (1990) through the use of tri-O-benzyl arabinosyl halides. Other C-5 aryl or alkyl substituted arabinosyl moieties may be synthesized, Danishefsky et al., J.Amer.Chem.Soc. 107:1274 (1985), Danishefsky, Aldrichimica Acta. 19:59-68 (1986) and references therein, and introduced into the disaccharide in the same manner. All of these references are incorporated herein by reference.

According to alternative Scheme IIa, the trisaccharide (A) is partially deprotected to furnish (D), which is subsequently reacted with the peracetylated sialic acid methyl ester (E) following a procedure described by Kameyama et al., XV Intl. Carbohyd. Symp., Abst. No. A096, (1990), and Carbihydrate Res., 209:c1-c4 (1991) (which are incorporated herein by reference), yielding (F) after chromatographic purification. Treatment of (F) sequentially with methylhydrazine, N-acetylation, O-deacetylation and ester hydrolysis furnishes SLX-R.

Preferred examples of R for scheme II and IIa include alkyl (straight chain, branched, saturated, mono- and poly-unsaturated); serine (D or L); serine containing peptides; di- and tri-alkanolamines (e.g. $[HO(CH_2)_n]_2NH$, $[HO(CH_2)_n]_3N$; wherein n = C_2-C_{20} as straight chain, branched, unsaturated, mono- and poly-unsaturated). R can also be aryl, substituted aryl (e.g., Me, OH, I; alone or in combination including ^{125}I), alkylaryl, arylalkyl or other moiety, as the skilled artisan would include for the desired use. The introduction of iodine into phenolic compounds such as tyrosine is known in the art. Radical groups containing phenols are useful for the introduction of ^{125}I radioisotope, yielding compounds which are useful in diagnosis.

Compounds comprising SLX and related structures may also be used to assay for the presence of other compounds which are capable of inhibiting intercellular adhesion mediated by selectins. A number of methods can be used to assay the biological activity of test compounds for the ability to inhibit the selectin-mediated response. Ideally, the assays of the present invention allow large scale in vitro or in vivo screening of a variety of compounds.

The agent or test compound to be screened will typically be a synthetic or naturally-produced biomolecule, such as a peptide, polypeptide, protein (e.g., monoclonal antibody), carbohydrate (e.g., oligosaccharide), glycoconjugate, nucleic acid, and the like. The compounds are synthetically produced using, for instance, the methods for synthesizing oligosaccharides described above (see, also, Khadem, Carbohydrate Chemistry (Academic Press, San Diego,CA, 1988), which is incorporated herein by reference). Methods for synthesizing polypeptides of defined composition are well known in the art (see, Atherton et al. Solid Phase Peptide Synthesis (IRL Press, Oxford, 1989) which is incorporated herein by reference). If the synthetic test compounds are polymeric (e.g., polypeptides or polysaccharides) they are preferably altered in a systematic way to identify the sequence of monomers which have the desired effect (see, e.g., U.S. Patent No. 4,833,092, which is incorporated herein by reference).

Test compounds may also be isolated from any natural source, such as animal, plant, fungal, or bacterial cells in accordance with standard procedures as described above. Potentially useful monoclonal antibodies can be prepared according to standard methods described in more detail, below.

The assays of the present invention are particularly useful in identifying compounds which act as antagonists or agonists of a ligand molecule. Antagonists are compounds which reverse the physiological effect of a ligand or exclude binding of the ligand to the receptor. An antagonist usually competes directly or indirectly with the ligand for the receptor binding site and, thus, reduces the proportion of ligand molecules bound to the receptor. Typically, an antagonist will be the topographical equivalent of the natural ligand and will compete directly with the ligand for the binding site on the selectin. Such a compound is referred to here as a "mimetic." An SLX mimetic is a molecule that conformationally and functionally serves as substitute for an SLX moiety in that it is recognized by a selectin receptor. Alternatively, if the ligand and the test compound can bind the receptor simultaneously, the compound may act non-competitively. A non-competitive inhibitor acts by decreasing or inhibiting the subsequent physiological effects of receptor-ligand interactions rather than by diminishing the proportion of ligand molecules bound to the receptor. Finally, the assays of the present invention can be used to identify synthetic or naturally occurring agonists, that is, compounds which bind the receptor and initiate a physiological response similar to that of the natural ligand.

Numerous direct and indirect methods for <u>in vitro</u> screening of inhibitors of ligand-receptor interactions are available and known to those skilled in the art. For instance, the ability to inhibit adhesion of SLX-bearing cells to cells expressing a particular selectin can be determined. As discussed above, selectin receptor genes have been cloned, thus the genes can be inserted and expressed in a wide variety of cells, such as COS cells, CHO cells and the like. In addition, cells which do not normally express SLX are capable of being transformed with one or more glycosyltransferase genes which

confer on the transformed cells the ability to synthesize the ligand. (see, e.g., Lowe et al., Cell 63:475-484 (1990), which is incorporated herein by reference.) Typically, the test compound or agent is incubated with labelled SLX-bearing cells and activated endothelial cells immobilized on a solid surface. Inhibition of cellular adhesion is then determined by detecting label bound to the surface after appropriate washes. In an exemplified assay described below, promyelocytic HL-60 cells and activated human endothelial cells or activated platelets are used.

Since a ligand specific for selectin receptors has now been identified, isolated ligand molecules can also be used in the assays. The terms "isolated selectin-binding agent" or "isolated SLX moiety" as used herein refer to a selectin binding compound that is in other than its native state, e.g., not associated with the cell membrane of a cell that normally expresses the ligand. Thus, an isolated SLX moiety may be a component of an isolated molecule, such as an oligosaccharide or a glycoconjugate. The isolated molecule may be synthesized or prepared from the membranes of SLX-bearing cells. Alternatively, the isolated selectin-binding agent or SLX moiety may be associated with a liposome or attached to a solid surface before use in the assay. Methods for preparing selectin-binding liposomes and for immobilizing various biomolecules are extensively discussed below.

Typically, the in vitro assays of the present invention are competition assays which detect the ability of a test compound to competitively inhibit binding of a compound known to bind either the receptor or the ligand. Inhibition of binding between SLX and a selectin receptor is usually tested. Inhibition of other binding interactions are also suitable, for instance, inhibition of the binding between a monoclonal antibody (e.g., CSLEX-1) and SLX or between an SLX mimetic and a selectin inhibitor can be used. Numerous types of competitive assays are known (see, e.g., U.S. Patents No. 3,376,110, 4,016,043, and Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Publications, N.Y. (1988), which are incorporated herein by reference).

The assays of the present invention are also suitable for measuring binding of a test compound to one component alone rather than using a competition assay. For instance, immunoglobulins can be used to identify compounds that contain the SLX moiety. Standard procedures for monoclonal antibody assays, such as ELISA, may be used (see, Harlow and Lane, supra). When assaying for glycolipids comprising the SLX antigen, the reactivity of the monoclonal antibody with the antigen can be assayed by TLC immunostaining by the method originally described in Magnani et al., Anal. Biochem. 109:399-402 (1980) or by solid-phase radioimmunoassay as described by Kanagi et al., Cancer Res. 43:4997-5005 (1983); which are incorporated herein by reference. Glycoproteins can be assayed by standard immunoblotting procedures as described in Harlow and Lane, supra. Sandwich assay formats are also suitable (see, e.g., U.S. Patent Nos. 4,642,285; 4,299,916; and 4,391,904; and Harlow and Lane, supra all of which are incorporated herein by reference). Typically, compounds which have been identified in a binding assay will be further tested to determine their ability to inhibit receptor-ligand interactions.

Other assay formats involve the detection of the presence or absence of various physiological changes in either ligand-bearing or selectin-bearing cells that result from the interaction. Examples of suitable assays include the measurement of changes in transcription activity induced by binding (see, e.g., EPO Publication No. 3712820), the detection of various cell mediated extra-cellular effects (see, e.g., PCT Publication No. 90/00503), and the detection of changes in the membrane potential of individual cells (see, e.g., U.S. Patent No. 4,343,782), all of which are incorporated herein by reference. Alternatively, conformational changes in isolated receptors or ligands can be detected; see, e.g., U.S. Patent No. 4,859,609, which is incorporated herein by reference.

Any component of the assay, including the ligand, the receptor, or the test compound, may be bound to a solid surface. Many methods for immobilizing biomolecules on solid surfaces are known in the art. For instance, the solid surface

may be a membrane (e.g., nitrocellulose), a microtiter dish (e.g., PVC or polystyrene) or a bead. The desired component may be covalently bound or noncovalently attached through unspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, etc. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cermets or the like. In addition are included substances that form gels, such as proteins, e.g., gelatins, lipopolysaccharides, silicates, agarose and polyacrylamides or polymers which form several aqueous phases, such as dextrans, polyalkylene glycols (alkylene of 2 to 3 carbon atoms) or surfactants e.g. amphiphilic compounds, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts and the like. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be employed to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. See for example Immobilized Enzymes, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas, J.

Biol. Chem. 245 3059 (1970) which is incorporated herein by reference.

In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labelled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labelled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082, which are incorporated herein by reference.

Many assay formats employ labelled assay components such as SLX ligands, SLX mimetics, immunoglobulins, receptors, or test compounds. The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. A wide variety of labels may be used. The component may be labelled by any one of several methods. The most common method of detection is the use of autoradiography with ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P labelled compounds or the like. The choice of radioactive isotope depends on research preferences due to ease of synthesis, varying stability, and half lives of the selected isotopes. Other non-radioactive labels include ligands which bind to labelled antibodies, fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as specific binding pair members for a labelled ligand. The choice of label depends on sensitivity required, ease of conjugation with the compound, stability requirements, and available instrumentation.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (e.g., streptavidin) molecule which is either

inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. Ligands and anti-ligands may be varied widely. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labelled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by reference.

As discussed above, in addition to various inhibitor compounds which comprise an accessible SLX unit or SLX mimetic, the present invention also provides monoclonal antibodies capable of inhibiting intercellular adhesion mediated by selectins as well as methods for identifying such antibodies. The monoclonal antibodies bind a selectin ligand or receptor and block cellular adhesion. Thus, the multitude of techniques available to those skilled in the art for production and manipulation of various immunoglobulin molecules can be applied to inhibit intercellular adhesion.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. The immunoglobulins may exist in a variety of forms besides antibodies, including for example, Fv, Fab, and $F(ab)_2$, as well as in single chains

(e.g., Huston et al., Proc. Nat. Acad. Sci. U.S.A. 85:5879-5883 (1988) and Bird et al., Science 242:423-426 (1988), which are incorporated herein by reference). (see, generally, Hood et al., Immunology, 2nd ed., Benjamin, N.Y. (1984), and Hunkapiller and Hood, Nature 323:15-16 (1986), which are incorporated herein by reference.)

Antibodies which bind the SLX antigen may be produced by a variety of means. The production of non-human monoclonal antibodies, e.g., murine, lagomorpha, equine, etc., is well known and may be accomplished by, for example, immunizing the animal with the SLX antigen or a preparation containing a glycoprotein or glycolipid comprising the antigen. Antibody-producing cells obtained from the immunized animals are immortalized and screened, or screened first for the production of antibody which inhibits the interaction of the viral surface protein with the receptor molecule and then immortalized. For a discussion of general procedures of monoclonal antibody production see Harlow and Lane, Antibodies, A Laboratory Manual (1988), supra.

The generation of human monoclonal antibodies to a human antigen (in the case of an SLX unit isolated from human tissue) may be difficult with conventional techniques. Thus, it may be desirable to transfer the antigen binding regions of the non-human antibodies, e.g., the $F(ab')_2$ or hypervariable regions, to human constant regions (Fc) or framework regions by recombinant DNA techniques to produce substantially human molecules. Such methods are generally known in the art and are described in, for example, U.S. 4,816,397, EP publications 173,494 and 239,400, which are incorporated herein by reference. Alternatively, one may isolate DNA sequences which encode a human monoclonal antibody or portions thereof that specifically bind to the human SLX by screening a DNA library from human B cells according to the general protocol outlined by Huse et al., Science 246:1275-1281 (1989), incorporated herein by reference, and then cloning and amplifying the sequences which encode the antibody (or binding fragment) of the desired specificity.

A number of currently available monoclonal antibodies can be used according to the present invention to inhibit intercellular adhesion mediated by selectins. For instance, CSLEX-1 (see, Campbell et al., J. Biol. Chem. 259:11208-11214 (1984)), VIM-2, which recognizes a sequence slightly different from SLX (see, Macher et al., supra), FH6 (described in U.S. Patent No. 4,904,596) (all references are incorporated herein by reference) or SH_3 and SH_4 generated by Dr. S. Hakomori of the Biomembrane Institute in Seattle, Washington.

The compounds of the present invention, including immunoglobulins, can be used in preparing pharmaceutical formulations as discussed below. If the compound is an oligosaccharide or glycoconjugate, the SLX or SLX-mimetic moiety can be presented in a variety of forms, but should be able to effectively bind to a selectin receptor, such as ELAM-1, GMP-140, or MEL-14 antigen and thereby inhibit intercellular adhesion.

The pharmaceutical compositions of the present invention can be used to block or inhibit cellular adhesion associated with a number of disorders. For instance, a number of inflammatory disorders are associated with selectins expressed on vascular endothelial cells and platelets. The term "inflammation" is used here to refer to reactions of both the specific and non-specific defense systems. A specific defense system reaction is a specific immune system reaction to an antigen. Example of specific defense system reactions include antibody response to antigens, such as viruses, and delayed-type hypersensitivity. A non-specific defense system reaction is an inflammatory response mediated by leukocytes generally incapable of immunological memory. Such cells include macrophages, eosinophils and neutrophils. Examples of non-specific reactions include the immediate swelling after a bee sting, and the collection of PMN leukocytes at sites of bacterial infection (e.g., pulmonary infiltrates in bacterial pneumonias and pus formation in abscesses).

Other treatable disorders include, e.g., rheumatoid arthritis, post-ischemic leukocyte-mediated tissue damage (reperfusion injury), frost-bite injury or shock, acute

leukocyte-mediated lung injury (e.g., adult respiratory distress syndrome), asthma, traumatic shock, septic shock, nephritis, and acute and chronic inflammation, including atopic dermatitis, psoriasis, and inflammatory bowel disease. Various platelet-mediated pathologies such as atherosclerosis and clotting can also be treated. In addition, tumor metastasis can be inhibited or prevented by inhibiting the adhesion of circulating cancer cells. Examples include carcinoma of the colon and melanoma.

By way of example, reperfusion injury is particularly amenable to treatment by compositions of the present invention. Compositions which inhibit a GMP-140 selectin-ligand interaction may be particularly useful for treating or preventing reperfusion injury. The present invention may be used prophylactically prior to heart surgery to enhance post-surgical recovery.

Because GMP-140 is stored in Weibel-Palade bodies of platelets and endothelial cells and is released upon activation by thrombin to mediate adhesion of neutrophils and monocytes, inhibitors of the GMP-140 -ligand interaction may be especially useful in minimizing tissue damage which often accompanies thrombotic disorders. For instance, such inhibitors may be of therapeutic value in patients who have recently experienced stroke, myocardial infarctions, deep vein thrombosis, pulmonary embolism, etc. The compounds are especially useful in pre-thrombolytic therapy.

Compositions of the invention find particular use in treating the secondary effects of septic shock or disseminated intravascular coagulation (DIC). Leukocyte emigration into tissues during septic shock or DIC often results in pathological tissue destruction. Furthermore, these patients may have widespread microcirculatory thrombi and diffuse inflammation. The therapeutic compositions provided herein inhibit leukocyte emigration at these sites and mitigates tissue damage.

The inhibitors of selectin-ligand interaction also are useful in treating traumatic shock and acute tissue injury associated therewith. Because the selectins play a role in

recruitment of leukocytes to the sites of injury, particularly ELAM-1 in cases of acute injury and inflammation, inhibitors thereof may be administered locally or systemically to control tissue damage associated with such injuries. Moreover, because of the specificity of such inhibitors for sites of inflammation, e.g., where ELAM-1 receptors are expressed, these compositions will be more effective and less likely to cause complications when compared to traditional anti-inflammatory agents.

Thus, the present invention also provides pharmaceutical compositions which can be used in treating the aforementioned conditions. The pharmaceutical compositions are comprised of biomolecules or other compounds which comprise an SLX unit, antibodies which bind to SLX, or other compounds which inhibit the interaction between the SLX ligand and selectin receptors, together with pharmaceutically effective carriers. A biomolecule of the present invention may be a peptide, polypeptide, protein (e.g., an immunoglobulin), carbohydrate (e.g., oligosaccharide or polysaccharide), glycoconjugate (e.g., glycolipid or glycoprotein), nucleic acid, and the like. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see, Langer, Science 249:1527-1533 (1990), which is incorporated herein by reference.

In light of the complexity of the inflammatory response in mammals, one of skill will readily recognize that the pharmaceutical compositions of the present invention may comprise SLX bearing compounds in admixture with other compounds known to interfere with the function of other cellular adhesion molecules. For instance, members of the integrin family of adhesion molecules are thought to play a role in the extravasation of leukocytes at points of infection. For a review of intercellular adhesion receptors, including selectin receptors, and their role immune function, see Springer, Nature 346:425-434 (1990), which is incorporated herein by reference. In addition, successful treatment using the pharmaceutical compositions of the present invention may

also be determined by the state of development of the condition to be treated. Since different adhesion molecules may be up or down regulated in response to a variety of factors during the course of the disease or condition, one of skill will recognize that different pharmaceutical compositions may be required for treatment of different inflammatory states.

In one embodiment, the SLX ligand of the pharmaceutical composition can be used to target conventional anti-inflammatory drugs or other agents to specific sites of tissue injury. By using a selectin-binding moiety such as an SLX ligand or SLX mimetic to target a drug to a selectin receptor on, e.g., a vascular endothelial cell, such drugs can achieve higher concentrations at sites of injury. Side effects from the conventional anti-inflammatory chemotherapeutic agents can be substantially alleviated by the lower dosages, the localization of the agent at the injury sites and/or the encapsulation of the agent prior to delivery.

The targeting component, i.e., the SLX ligand or an SLX mimetic which binds to a desired selectin, can be directly or indirectly coupled to the chemotherapeutic agent. The coupling, which may be performed by means, generally known in the art, should not substantially inhibit the ability of the ligand to bind the receptor nor should it substantially reduce the activity of the chemotherapeutic agent. A variety of chemotherapeutics can be coupled for targeting. For example, anti-inflammatory agents which may be coupled include SLX-bearing compounds of the present invention, immunomodulators, platelet activating factor (PAF) antagonists, cyclooxygenase inhibitors, lipoxygenase inhibitors, and leukotriene antagonists. Some preferred moieties include cyclosporin A, indomethacin, naproxen, FK-506, mycophenolic acid, etc. Similarly, anti-oxidants, e.g., superoxide dismutase, are useful in treating reperfusion injury when targeted by a SLX ligand or mimetic. Likewise, anticancer agents can be targeted by coupling the SLX ligand or mimetic to the chemotherapeutic agent. Examples of agents which may be coupled include daunomycin, doxorubicin, vinblastine, bleomycin, etc.

The selectin receptor targeting may also be accomplished via amphipaths, or dual character molecules (polar:nonpolar) which exist as aggregates in aqueous solution. Amphipaths include nonpolar lipids, polar lipids, mono- and diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids and salts. These molecules can exist as emulsions and foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions and lamellar layers. These are generically referred to herein as liposomes. In these preparations the drug to be delivered is incorporated as part of a liposome in conjunction with a SLX ligand or mimetic which binds to the selectin receptor. Thus, liposomes filled with a desired chemotherapeutic agent can be directed to a site of tissue injury by the selectin-SLX ligand interaction. When the liposomes are brought into proximity of the affected cells, they deliver the selected therapeutic compositions.

The liposomes of the present invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size and stability of the liposomes in the bloodstream.

Typically, the major lipid component in the liposomes is phosphatidylcholine. Phosphatidylcholines having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In general, less saturated phosphatidylcholines are more easily sized, particularly when the liposomes must be sized below about 0.3 microns, for purposes of filter sterilization. Methods used in sizing and filter-sterilizing liposomes are discussed below. The acyl chain composition of phospholipid may also affect the stability of liposomes in the blood. One preferred phosphatidylcholine is partially hydrogenated egg phosphatidylcholine.

Targeting of liposomes using a variety of targeting agents (e.g., ligands, receptors and monoclonal antibodies) is well known in the art. (see, e.g., U.S. Patent Nos. 4,957,773 and 4,603,044, both of which are incorporated herein by

reference). Glycoproteins and glycolipids of a variety of molecular weights can be used as targeting agents. Typically, glycoproteins having a molecular weight less than about 300,000 daltons, preferably between about 40,000 and about 250,000 are used, more preferably between about 75,000 and about 150,000. Glycolipids of molecular weight of less than about 10,000 daltons, preferably between about 600 and about 4,000 are used.

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, such as phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid derivatized bleomycin.. Antibody targeted liposomes can be constructed using, for instance, liposomes which incorporate protein A (see, Renneisen, et al., J. Biol. Chem., 265:16337-16342 (1990) and Leonetti et al., Proc. Natl. Acad. Sci. (USA) 87:2448-2451 (1990), both of which are incorporated herein by reference).

Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target agents are available for interaction with the selectin receptor. The liposome is typically fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion which is firmly embedded and anchored in the membrane. It must also have a hydrophilic portion which is chemically available on the aqueous surface of the liposome. The hydrophilic portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent which is added later. Therefore, the connector molecule must have both a lipophilic anchor and a hydrophilic reactive group suitable for reacting with the target agent and holding the target agent in its correct position, extended out from the liposome's surface. In some cases it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking the connector molecule which is

in the membrane with the target agent which is extended, three dimensionally, off of the vesicle surface.

Liposome charge is an important determinant in liposome clearance from the blood, with negatively charged liposomes being taken up more rapidly by the reticuloendothelial system (Juliano, Biochem. Biophys. Res. Commun. 63:651 (1975)) and thus having shorter half-lives in the bloodstream. Liposomes with prolonged circulation half-lives are typically desirable for therapeutic and diagnostic uses. Liposomes which can be maintained from 8, 12, or up to 24 hours in the bloodstream provide sustained release of the selectin-ligand inhibitors of the invention, or may facilitate targeting of the inhibitors (which may be labelled to provide for in vivo diagnostic imaging) to a desired site before being removed by the reticuloendothelial system.

Typically, the liposomes are prepared with about 5-15 mole percent negatively charged phospholipids, such as phosphatidylglycerol, phosphatidylserine or phosphatidylinositol. Added negatively charged phospholipids, such as phosphatidylglycerol, also serves to prevent spontaneous liposome aggregating, and thus minimize the risk of undersized liposomal aggregate formation. Membrane-rigidifying agents, such as sphingomyelin or a saturated neutral phospholipid, at a concentration of at least about 50 mole percent, and 5-15 mole percent of monosialylganglioside, may provide increased circulation of the liposome preparation in the bloodstream, as generally described in U.S. Pat. No. 4, 837,028, incorporated herein by reference.

Additionally, the liposome suspension may include lipid-protective agents which protect lipids and drug components against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxianine, are preferred.

A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Pat. Nos. 4, 235,871, 4,501,728 and 4,837,028, incorporated herein by reference. One

method produces multilamellar vesicles of heterogeneous sizes. In this method, the vesicle forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form. This film is covered with an aqueous solution of the targeted drug and the targeting component and allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate.

The hydration medium contains the targeted drug at a concentration which is desired in the interior volume of the liposomes in the final liposome suspension. Typically the drug solution contains between 10-100 mg/ml in a buffered saline. The concentration of the targeting SLX molecule or mimetic which binds a selectin is generally between about 0.1 - 20 mg/ml.

Following liposome preparation, the liposomes may be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. One preferred size range is about 0.2-0.4 microns, which allows the liposome suspension to be sterilized by filtration through a conventional filter, typically a 0.22 micron filter. The filter sterilization method can be carried out on a high through-put basis if the liposomes have been sized down to about 0.2-0.4 microns.

Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a

standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination.

Extrusion of liposome through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing liposome sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

Even under the most efficient encapsulation methods, the initial sized liposome suspension may contain up to 50% or more drug and targeting agent in free (non-encapsulated) form. Therefore, to maximize the advantages of liposomal targeted drug, it is important to remove free drug and targeting agent from the final injectable suspension.

Several methods are available for removing non-entrapped compound from a liposome suspension. In one method, the liposomes in the suspension are pelleted by high-speed centrifugation leaving free compound and very small liposomes in the supernatant. Another method involves concentrating the suspension by ultrafiltration, then resuspending the concentrated liposomes in a drug-free replacement medium. Alternatively, gel filtration can be used to separate large liposome particles from solute molecules.

Following treatment to remove free drug and/or targeting agent, the liposome suspension is brought to a desired concentration for use in intravenous administration. This may involve resuspending the liposomes in a suitable volume of injection medium, where the liposomes have been concentrated, for example by centrifugation or ultrafiltration, or concentrating the suspension, where the drug removal step has increased total suspension volume. The suspension is then sterilized by filtration as described above. The liposome-ligand preparation may be administered parenterally or locally in a dose which varies according to, e.g., the manner of

administration, the drug being delivered, the particular disease being treated, etc.

For pharmaceutical compositions which comprise the SLX ligand, and/or SLX mimetics which bind to selectin receptors, the dose of the compound will vary according to, e.g., the particular compound, the manner of administration, the particular disease being treated and its severity, the overall health and condition of the patient, and the judgment of the prescribing physician. For example, for the treatment of reperfusion injury, the dose is in the range of about 50 μg to 2,000 mg/day for a 70 kg patient. Ideally, therapeutic administration should begin as soon as possible after the myocardial infarction or other injury. The pharmaceutical compositions are intended for parenteral, topical, oral or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and dragees.

Preferably, the pharmaceutical compositions are administered intravenously. Thus, this invention provides compositions for intravenous administration which comprise a solution of the compound dissolved or suspended in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride,

calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of SLX ligand or mimetic, which may be combined with other SLX ligands or mimetics to form a "cocktail" for increased efficacy in the pharmaceutical formulation, can vary widely, i.e., from less than about 0.05%, usually at or at least about 1% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. The cocktail may also comprise a monoclonal antibody which binds to selectin receptor, e.g., a monoclonal antibody to ELAM-1 or GMP-140, combined with the SLX ligand, a ligand mimetic or a monoclonal antibody to the ligand, so as to effectively inhibit the ligand-receptor interaction. As described above, the cocktail components may be delivered via liposome preparations.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 25 mg of the compound. Actual methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, PA (1985), which is incorporated herein by reference.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more SLX ligands or mimetics of the invention, preferably about 20% (see, Remington's, supra).

For aerosol administration, the compounds are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of SLX

oligosaccharide ligands or mimetics are 0.05% - 30% by weight, preferably 1% - 10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquefied propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above may also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

The compositions containing the compounds can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient, but generally range from about 0.5 mg to about 2,000 mg of SLX oligosaccharide or SLX mimetic per day for a 70 kg patient, with dosages of from about 5 mg to about 200 mg of the compounds per day being more commonly used.

In prophylactic applications, compositions containing the compounds of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, but generally range from about 0.5 mg to about 1,000 mg per 70 kilogram patient, more commonly from about 5 mg to about 200 mg per 70 kg of body weight.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of SLX oligosaccharide or SLX mimetic of this invention sufficient to effectively treat the patient.

The compounds may also find use as diagnostic reagents. For example, labeled compounds can be used to locate areas of inflammation or tumor metastasis in a patient suspected of having an inflammation. For this use, the compounds can be labeled with ^{125}I, ^{14}C, or tritium.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE I

Isolation of α1,3-fucosyltransferase I from Golgi Apparatus

LEC11, HL-60, HT-29, certain adenocarcinomas (colo 205 cells in particular), and polymorphonuclear leukocytes (PMN, neutrophils) contain a very specific α1,3-fucosyltransferase I, which is able to transfer fucose from GDP-fucose to the sialylated substrates NeuAcα2,3Galβ1,4GlcNAc or NeuGcα2,3Galβ1,4GlcNAc.

It is well known in the art that fucose is transferred to oligosaccharide chains in the lumen of the Golgi apparatus via specific fucosyltransferases, reviewed in Schacter and Roseman, in "The Biochemistry of Glycoproteins and Proteoglycans", W. Lennarz, ed., Plenum Press, New York, pp. 85-160 (1980), which is incorporated herein by reference. Since the subcellular localization of the fucosyltransferases

is in the Golgi apparatus, the first step in the isolation of these enzymes is to isolate a Golgi apparatus fraction from a cell line which expresses this novel and specific α1,3-fucosyltransferase.

Golgi apparatus-derived vesicle fractions are prepared by a modification of the procedure described by Balch et al., Cell, 39:405 (1984) which is incorporated herein by reference. The LEC11, HL-60, HT-29, PMN, colo 205 or other cell lines containing the α1,3-fucosyltransferase I are grown in suspension to a density of approximately 5 X 10^5 cells/ml. Cells are harvested from the suspension culture by centrifugation at 2,000 X g. The resulting cell pellet from a 12 liter suspension (6 X 10^9 cells) is resuspended in 3 volumes (packed cell volume) of ice-cold 0.25M sucrose (w/v) solution containing Tris-Cl (10mM), pH 7.0, heat inactivated fetal calf serum (7%), and Aprotinin (100 μg/ml, Sigma Chemical, Co. St. Louis, Mo.).

The cells are disrupted (approximately 60 strokes) with a tight fitting Wheaton glass dounce homogenizer using the A pestle. The homogenate is centrifuged for 5 min. at 500 X g in a table-top clinical centrifuge. Lipid and insoluble material remaining at the top of the solution in the centrifuge tube is discarded. The cloudy supernatant is transferred to a clean tube, and the sucrose concentration of the supernatant fraction is then adjusted to 40% (w/v) sucrose in Tris-Cl (20 mM), pH 7.0, with the aid of a refractometer. Five milliliters of this suspension is transferred to an ultracentrifuge tube and is layered sequentially with 2.5 ml of 35% (w/v) sucrose in Tris-Cl (10 mM, pH 7.0) and 2.0 ml of 29% (W/v) sucrose in 10 mM Tris-Cl buffer. The gradient is centrifuged for 1 hr. at 110,000 X g in a SW-41 rotor (Beckman) at 5°C. Golgi apparatus enriched vesicles are collected from the 29% to 35% sucrose interphase. Other subcellular fractions are found at other interphases in the gradient; e.g., vesicles derived from the rough and smooth endoplasmic reticulum band below the Golgi derived vesicles, etc. The band removed from the 29% to 35% interphase is analyzed for the presence and amount of sialyltransferase activity.

The enzyme sialytransferase is only known to be found within Golgi apparatus-derived vesicles and is used by those trained in the art as a marker to assess the authenticity of the band collected from the 29-35% interphase. Sialyltransferase assays are performed using asialofetuin as the acceptor as described by Briles et al., J. Biol. Chem., 252:1107 (1977). A good Golgi apparatus derived vesicle preparation from LEC cells typically has a sialyltransferase-specific activity of 3.0 nmole/mg protein/hr.

The resulting Golgi apparatus preparation is then used as a source of the α1,3-fucosyltransferase I used in the enzymatic synthesis described above.

EXAMPLE II

Demonstration of Intercellular Adhesion by Cells Expressing SLX

The ability of LEC11 cells (which express SLX) to bind to activated endothelial cells expressing ELAM-1 was compared to that of CHO cells and another glycosylation mutant, LEC12, which expresses the structure Le^x, a non-sialylated form of SLX.

MATERIALS

Passage 5 human umbilical vein endothelial cells (HUVEC) (Clonetics) which had been grown on a gelatin coated 48 well assay plate were used as the source of endothelial cells. Cells were stimulated with IL-1β (Genzyme) at 30 μg/ml. Cells were stimulated for exactly 4 hrs. HL-60 cells provided by American Type Culture Collection (ATCC No. CCL 240) were used as the source of control ligand bearing cells. These were harvested from bulk culture in RPMI 1640 (Gibco) containing Penicillin (100 units/ml)/ Streptomycin (100 Mcg/ml)(Irvine Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10% Fetal Bovine Serum (Hazleton) (hereafter referred to as CRPMI). LEC11, LEC12 and CHO-K1 were provided by Dr. P. Stanley. They were grown in suspension culture in complete alpha MEM containing ribonucleotides and deoxyribonucleotides (Gibco), Penicillin (100 units/ml)/ Streptomycin (100 μg/ml)(Irvine

Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10% Fetal Bovine Serum (Hazelton).

PROCEDURE

1. HL-60, LEC11, LEC12 and CHO-K1 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 3 x 10^6 cells of each type were pelleted in a 10 ml test tube and 300 μl of ^{51}Cr (450 μCi) (New England Nuclear) was added to each pellet. The tubes were allowed to incubate 1 hour at 37°C with gentle agitation.

2. Labeled cells were washed 3X in medium and resuspended to 2 x 10^5/ 400 μl (6ml). The tubes were then placed in a 4°C ice bath.

3. After 4 hours incubation with IL-1β the assay plate containing activated HUVEC was removed from the incubator and chilled for 15 minutes by placing the plate in a 4°C ice bath.

4. When the temperature in both samples had equilibrated, the medium was removed from the assay wells with a pasteur pipette a few wells at a time.

5. Labeled cells were added to the wells in 400 μl volumes equal to 2 x 10^5 cells/well. Three 400 μl aliquots of each cell suspension were placed in glass tubes for determination of input CPMs.

6. The plate was incubated in the ice water bath for 30 minutes.

7. Unbound cells were removed from the wells of the assay plate by systematic resuspension using a pasteur pipette followed by addition and removal of 0.7 ml of medium.

8. All of the medium was removed from the wells and a solution of 0.125 M Tris, 2% SDS and 10% glycerin was added

(0.3 ml). The plate was allowed to stand for 30 minutes and then 0.5 ml of dH_2O was added to each well.

9. The fluid in each well was resuspended with a P1000 pipette and transferred to a glass test tube. The P1000 tip was ejected into the tube.

10. The tubes, including those containing the input CPM samples were counted in a gamma counter.

11. CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. The mean and standard deviation of triplicate assay points were plotted.

The results obtained in this experiment, shown in Fig. 1, indicate that cells expressing SLX have the ability to bind effectively to activated vascular endothelial cells expressing ELAM-1. These data show that LEC11 cells which express high levels of the unique carbohydrate SLX bind exceptionally well to IL-1β activated HUVEC, while LEC12 and CHO-K1 which lack significant quantities of this carbohydrate are poor binders of the activated HUVEC. This conclusion is further supported by the observation that this binding occurs at 4°C, a characteristic of ELAM-1 mediated binding.

EXAMPLE III

Inhibition of Intercellular Adhesion by Monoclonal Antibodies Specific for SLX.

Two sets of experiments are described which confirm that the ligand on neutrophils for ELAM-1 contains an oligosaccharide where the terminal sugars are NeuAcα2,3Galβ1,4(Fuc α1,3)GlcNac(SLX).

These experiments are performed by assaying the ability of monoclonal antibodies specific for sialylated Le^x and for the unsialylated form, Le^x, to block the ELAM-1 mediated adhesion of HL-60 cells to IL-1β stimulated HUVEC.

A. Monoclonal Antibody Panel 1

Materials: Passage 3 HUVEC from cultures initiated for the present experiments were used as described above. Two sets of triplicate wells were left unstimulated as controls. Four triplicates were stimulated with IL-1β (Genzyme) at 10 μg/ml and 4 at 20 μg/ml. Cells were stimulated for exactly 4 hours. HL-60 cells obtained from the American Type Culture Collection were used as the source of ligand bearing cells. These were harvested from bulk culture in RPMI-1640 (Gibco) containing penicillin (100 units/ml), streptomycin (100 μg/ml) (Irvine Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10% Fetal Bovine Serum (Hazleton) (hereafter referred to as cRPMI).

Monoclonal antibody preparations included SNH3 (IgM) at about 20 μg/ml and SH1 (IgG3) at about 10 μg/ml. The specificity of SNH3 is for SLX, while SH1 recognizes the unsialylated structure.

Procedure:

1. HL-60 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 3 x 10^6 cells were placed in each of 2, 10 ml test tubes and 300 μl of ^{51}Cr (450 μCi) (New England Nuclear) was added to each tube. The tubes were allowed to incubate 1 hour at 37°C with gentle agitation.

2. The antibodies were supplied as hybridoma culture supernatants and contained 0.01% NaN3 and 0.05% thimerosal. To remove these preservatives, 5 ml. of each antibody was dialysed against 3 changes of 500 ml each of outdated tissue culture medium over 72 hours.

3. Antibodies were collected from dialysis and 3.5 ml of each was placed in 10 ml tubes. The remainder was retained for use in an ELISA assay for HL-60 binding. 7 ml of RPMI 1640 5% FCS was placed in a 4th tube for use as a control.

4. Labeled HL-60 cells were washed 3X in CRPMI and pooled into one tube. They were then centrifuged and resuspended to 1 ml in medium.

5. 200 μl of cell suspension was added to each of the antibody containing tubes and 400 μl to the control tube. Tubes were incubated 20 min. at 37°C with gentle agitation.

6. The stimulated HUVEC assay plate was removed from the incubator and the medium was removed from the wells with a pasteur pipette, a few wells at a time.

7. 0.5 ml of cell suspension was added to each of triplicate wells. Control cells were plated on unstimulated and stimulated HUVEC at both IL-1β concentrations. Test cells were added to stimulated wells only.

8. 0.5 ml aliquots of each cell suspension were added to glass tubes to be used to determine the input CPMs.

9. The assay plate was returned to the incubator (5% CO_2, 37°C) for 30 min.

10. An aliquot of each cell suspension was mixed with an equal volume of trypan blue and the cells were examined microscopically for viability. The results were: Control = 98%, SH1 = 92%, and SNH3 = 99%.

11. Unbound cells were removed from the wells of the assay plate by systematic resuspension using a pasteur pipette followed by addition and removal of 0.7 ml of medium.

12. All of the medium was removed from the wells and a solution of 0.125 M Tris, 2% SDS (Bio-Rad) and 10% glycerin (Fisher) was added (0.3 ml). The plates were allowed to stand for 30 min. and then 0.6 ml of dH_2O was added to each well.

13. The fluid in each well was resuspended with a P1000 pipette and transferred to a glass test tube. The P1000 tip was ejected into the tube.

14. The tubes, including those containing the input counts per minute (CPM) samples were counted in a gamma counter.

15. CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. The mean and standard deviation of triplicate assay points were plotted.

Replicates were judged to be best in the experiment in which high IL-1β was used to induce the endothelial cells.

The results showed that the monoclonal antibody SNH3 blocked the binding of HL-60 cells to IL-1β stimulated HUVEC via the ELAM-1 receptor. The control antibody SH1 which does not bind the SLX determinant did not block binding of HL-60 cells to ELAM-1. This suggests that the terminal sialic acid in the ligand is necessary for binding to ELAM-1.

B. Monoclonal Antibody Panel 2

Materials: Passage 3 HUVEC which had been grown on gelatin coated 48 well assay plates (Costar) were used as the source of endothelial cells. The plates were prepared as previously described above. Two sets of triplicate wells were left unstimulated as controls. Seven triplicates on each plate were stimulated with IL-1β at 30 μg/ml in 0.5 ml of EGM-UV. Cells were stimulated for exactly 4 hrs. HL-60 cells (ATCC) were used as the source of ligand bearing cells. These were harvested from bulk culture in CRPMI. Fresh hybridoma supernatants containing monoclonal antibodies included: FH6 (IgM) a lower affinity mAb; SNH-3 (IgM) (20 μg/ml); SH-1 (IgG_3) (10 μg/ml); FH-2 (IgM) a Le^x reactive mAb; SNH-4 (IgG3) a high affinity antibody; and CSLEX-1 (IgM) (provided by Dr. P. Terasaki, UCLA as purified immunoglobulin at 2.8 mg/ml, diluted to 9 μg/ml in Dulbecco's Modified Eagles Medium (DMEM) containing 5% FCS for use in this assay). The specificities of the antibodies were as follows: FH6, SNH-4, SNH3 and CSLEX-1 were specific for SLX; FH2 and SH1 were specific for the unsialylated Le^x.

Procedure:

1. HL-60 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 3 x 10^6 cells were placed in each of 2, 10 ml test tubes and 300 µl of ^{51}Cr (450 µCi) (New England Nuclear) was added to each tube. The tubes were allowed to incubate 1 hour at 37°C with gentle agitation.

2. Labeled HL-60 cells were washed 3X in DMEM containing 5% FCS (hereafter referred to as cDMEM) and pooled into one tube. They were then centrifuged and resuspended to 4 x 10^6 cells per ml in the same medium.

3. 3.2 ml of each monoclonal antibody culture supernatant, and 3.2 ml purified CSLEX-1 (29 µg), were added to separate test tubes; a control tube received 6.4 ml of medium.

4. 200 µl of cell suspension (equal to about 8 x 10^5 cells) was added to tubes containing the monoclonal antibodies and 400 µl to the control tube. Tubes were then incubated 20 min. at 37°C with gentle agitation.

5. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed one time with cDMEM and the medium was removed from the wells with a pasteur pipette, a few wells at a time.

6. 0.4 ml of cell suspension was added to each well of one of the two plates. Control cells were plated on unstimulated and stimulated HUVEC. Antibody treated cells were added to stimulated wells only.

7. 0.4 ml aliquots of each cell suspension were added to glass tubes to be used to determine the input CPMs.

8. The assay plate was incubated at 37°C for 30 min.

9. The remainder of each cell suspension and the assay plate were placed in an ice bath to chill for 20 min.

10. The cell suspensions were plated on the chilled plate as for the 37°C plate above. This plate was incubated for 30 min. at 40°C.

The remaining steps of the assay were performed as described in steps 11-15 of Section A above, except that in step 12 the plates were allowed to stand for 15 min. rather than 30 min.

The results, shown in Fig. 2A, indicate that the monoclonal antibodies SNH-3, FH6, SNH-4 and CSLEX-1, all specific for SLX, significantly blocked the binding of HL-60 cells to IL-1β stimulated HUVEC via the ELAM-1 receptor when incubated at 37°C. The monoclonal antibodies specific for Le^x (FH2 and SH1) were not effective inhibitors. Thus, the ligand for ELAM-1 contains the sialylated Le^x antigen or a similar structure found in cell surface glycoproteins or glycolipids.

When incubated at 4°C (Fig. 2B), antibodies FH6 and SNH-3 (both IgM's) enhanced binding. In these tests there appeared to be significant agglutination of the HL-60 cells in the wells, which may account for this observation.

C. Monoclonal Antibodies Block Adhesion of LEC11 Cells to Cells which Express ELAM-1

In this set of experiments the ability of monoclonal antibodies specific for SLX and for the unsialylated form, Le^x, to block the ELAM-1 mediated adhesion of LEC11 cells (which express SLX) and LEC12 cells (which express Le^x) to IL-1β stimulated HUVEC.

Materials: Passage 4 HUVEC served as the source of endothelial cells. The plates were prepared as previously described. Two sets of triplicate wells were left unstimulated as controls. 7 triplicates on each plate were stimulated with IL-1β at 30 μg/ml in a 0.5 ml volume of EGM-UV. Cells were stimulated for exactly 4 hrs. LEC11 and LEC12 cells, described generally in Stanley et al., J. Biol. Chem., 263:11374 (1988),

supra, were provided by Dr. P. Stanley. They were grown in suspension culture in complete alpha MEM containing ribonucleotides and deoxyribonucleotides (Gibco), penicillin (100 unitsl ml)/streptomycin (100 μg/ml) (Irvine Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10% FBS (Hazelton). The monoclonal antibodies used in these experiments are described in Section B, above. They included: FH6, SNH-3, SH-1, FH-2, SNH-4 and CSLEX-1.

Procedure:

1. LEC11 and LEC12 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 3 x 10^6 cells of each cell line were placed in each of 2, 10 ml test tubes and 300 μl of ^{51}Cr (450 μCi) (New England Nuclear) was added to each tube. The tubes were allowed to incubate 1 hr. at 37°C with gentle agitation.

2. The radiolabeled cells were washed X3 in cDMEM and pooled into one tube. They were then centrifuged and resuspended to 4 x 10^6 cells per ml in the same medium.

3. 1.6 ml of each monoclonal antibody supernatant, and 1.6 ml purified CSLEX-1 (15 μg), were added to separate test tubes; control tubes received 3.2 ml medium.

4. 200 μl of cell suspension equal to 4 x 10^5 LEC11 or LEC12 cells were added to tubes containing the monoclonal antibodies and 400 μl to the control tube. Tubes were incubated 20 min. at 37°C with gentle agitation.

5. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed one time with cDMEM and the medium was removed from the wells with a pasteur pipette, a few wells at a time.

6. The cell suspensions and the assay plate were placed in an ice bath to chill for 20 min.

7. 0.4 ml of cell suspension was added to each well of the previously described assay plate. Control cells were plated on unstimulated and stimulated HUVEC. Antibody treated cells were added to stimulated wells only. Each assay was done in triplicate.

8. 0.4 ml aliquots of each cell suspension were added to glass tubes to be used to determine the input CPMs.

9. The assay plate was incubated for 30 min. at 4°C.

The remaining steps of the assay were performed as described in steps 11-15 of Section A, above, except that in step 12 the plates were allowed to stand for 15 min.

The results shown, in Figs. 3A and 3B, indicate that the monoclonal antibodies SNH-3, FH6, SNH-4 and CSLEX-1 (all specific for SLX) significantly blocked the binding of LEC11 cells to IL-1β stimulated HUVEC via the ELAM-1 receptor. LEC12 cells, which do not express the SLX epitope, did not bind the activated endothelium. The monoclonal antibodies specific for Le^x (FH2 and SH1) caused minor inhibition of LEC11 binding.

Further confirmation that SLX is a primary ligand for ELAM-1 receptor was provided by removing sialic acid from LEC 11 and HL-60 cells. In these experiments the treatment of LEC 11 and HL-60 cells prior to adhesion assays with Clostridium perfringens neuraminidase (sialidase), 1.6 U/ml (Type X, Sigma Chem. Co.) for 90 min. at 37°C during ^{51}Cr-labelling. The results, shown in Fig. 4, confirm that sialidase substantially reduced the adhesion of LEC 11 and HL-60 cells by 70-85% to activated endothelial cells.

EXAMPLE IV

Liposomes of Glycosphingolipids Block Binding of SLX Cells to Activated Endothelial Cells

This Example describes the preparation of liposomes which contain various biosynthetically produced glycosphingolipids on which the terminal carbohydrate units are

either SLX, Le^x, or similar but not identical compounds. The ability of the liposomes which contain SLX or SLX mimetics to block the binding of SLX-expressing HL-60 cells and LEC11 cells to endothelial cells which have been stimulated to express ELAM-1 by treatment with IL-1β is shown.

Materials: The glycosphingolipids used in this experiment are shown in Table I; they were obtained from the Biomembrane Institute, Seattle, WA, and were either purified or biosynthetically produced and characterized by NMR and mass spectrometry, as generally described in Hakomori, S. I., et al., J. Biol. Chem., 259:4672 (1984), and Fukushi Y., et al., J. Biol. Chem. 259:10511 (1984), incorporated by referenced herein. S-diLe^x (SLX) was synthesized enzymatically by adding fucosyl residues using a colo 205 cell line as enzyme source and SH as substrate. Nonsialylated diLe^x was similarly synthesized using nLc6 as substrate and the cell line NCI H-69. See Holmes et al., J. Biol. Chem. 260:7619 (1985), incorporated by reference herein. SPG and SH were purified from bovine red blood cells, and nLc6 was produced by chemical removal of the terminal sialosyl residue from SH.

Table 1. Glycolipids tested for liposome inhibition of ELAM-1 mediated cell adhesion.

Generic	IUPAC	Structure
nLc_6	nLc_6	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
$diLe^1$	$III^3V^3Fuc_2nLc_6$	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ Fucα1 Fucα1
SPG	$IV^3NeuAcnLc_4$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
SH	$VI^3NeuAcnLc_6$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
$S\text{-}diLe^x$	$III^3V^3Fuc_2VI^3NeuAcnLc_6$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ Fucα1 Fucα1

PS\DOCS\6700

SUBSTITUTE SHEET

Liposomes containing the glycosphingolipids were formed as follows: 100 μg of glycolipid was added to 300 μg phosphatidylcholine (Sigma, egg yolk) and 500 μg cholesterol (Sigma) in chloroform-methanol (2:1) and the whole solution evaporated to dryness by N_2 in 15 ml screwcap tubes.

Passage 3 HUVEC, which had been grown on to confluence on a gelatin coated 48 well assay plate (Costar) were used as the source of endothelial cells. The plates were prepared as previously described. Two sets of triplicate wells were left unstimulated as controls. 14 triplicates were stimulated with IL-1β at 30 μg/ml in a 0.5 ml volume of EGM-UV. Cells were stimulated for exactly 4 hrs. HL-60 cells and LEC11 cells were cultured as described above.

Procedure:

1. One 48 well Costar cluster dish containing HUVEC grown to confluence on gelatin was removed from the incubator and the medium in each well was removed with a pasteur pipette and replaced either with 0.5 ml fresh EGM-UV medium or with the same medium containing 30 μg/ml IL-1β, and the plate then returned to the incubator for 4 hrs.

2. HL-60 cells and LEC11 cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue. 6×10^6 cells of each cell type were radiolabeled as follows: 3×10^6 cells of each type were placed in each of 2, 10 ml test tubes and 300 μl of ^{51}Cr (450 μCi) (New England Nuclear) was added to each tube. The tubes were allowed to incubate 1 hr. at 37°C with gentle agitation.

3. Radiolabeled HL-60 and LEC11 cells were washed 3X in CRPMI and pooled into one tube. They were then centrifuged and resuspended to 2×10^6 cells per ml in the same medium.

4. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed two times with RPMI 1640 containing 5 mg/ml bovine serum albumin (BSA).

5. Liposomes were prepared as follows: The evaporated pellets were dissolved in 100 μl of absolute ethanol and sonicated for 2 min. Two ml of PBS was added slowly to the tubes over two minutes while continuing to sonicate. This stock was diluted 1:10 in RPMI 1640 medium just prior to use and 50 μl of a stock solution of BSA at 100 mg/ml was added to each 1 ml of diluted liposomes to make a final concentration of 5 mg/ml BSA.

6. The medium was removed from the wells of the assay plate with a pasteur pipette, a few wells at a time, and 0.3 ml of liposome suspension was added to each of 6 IL-1β stimulated assay wells. Control wells received the liposome buffer containing ethanol, RPMI 1640 and BSA at the same concentrations as in the liposome containing wells. Control buffer was plated on unstimulated and stimulated HUVEC. Liposome containing samples were added to stimulated wells only.

7. The plates were incubated for 40 min. at 37°C and then 50 μl of ^{51}Cr labeled HL-60 or LEC11 cells were added to the assay wells. Each cell line was assayed in triplicate on each liposome preparation. The final concentration of cells was 10^5/350 μl/well. Three aliquots of 50 μl of each cell suspension were added to glass tubes to be used to determine the input CPMs, and the assay plate incubated at 37°C for 30 min.

8. Unbound cells were removed from the wells of the assay plates by systematic resuspension using a pasteur pipette followed by addition and removal of 0.7 ml of medium. All of the medium was removed from the wells and a solution of 0.125 M Tris, 2% SDS and 10% glycerin was added (0.3 ml). The plates were allowed to stand for 15 min. and then 0.5 ml of dH_2O was added to each well.

9. The fluid in each well was resuspended and transferred to a glass test tube. The pipette tip was ejected

into the tube. The tubes, including those containing the input CPM samples, were counted in a gamma counter. CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. The means and standard error of triplicate assay points were plotted.

As shown in Fig. 5, liposomes containing selected glycolipids having terminal sequences which contained SLX (S-diLe^x, Table 1) dramatically inhibited adhesion of HL-60 cells to activated endothelial cells at 4°C. Liposomes containing glycolipids with Le^x (di-Le^x) or other related carbohydrate structures (Table 1) exhibited minimal inhibition that was not dependent on the structure of the carbohydrate group. Similar results were obtained with LEC 11 cell adhesion. When the experiments were performed at 37°C, HL-60 cell adhesion was reduced by liposomes containing glycolipids with the SLX structure (S-diLe^x, 70%), and also to a lesser extent by liposomes containing Le^x (di-Le^x, 40%) suggesting that Le^x may also interact with ELAM-1, but with a lower affinity. These experiments show that biosynthetically produced SLX or similar SLX mimetic compounds when formulated into liposome compositions can serve as therapeutic compounds for, e.g., the reduction of leukocyte infiltration into inflammatory sites.

Jurkat cells bind to IL-1 activated endothelial cells predominantly through the V-CAM (endothelial cell) - VLA-4 (Jurkat cell) adhesion pair (Wayner et al., J. Cell Biol. 109:1321), in contrast to the adhesion of HL-60 and LEC 11 cells to activated endothelial cells through the ELAM-1 receptor. Jurkat cell adhesion was not inhibited by liposomes which contained SLX, but was completely inhibited by monoclonal antibody to the α subunit of the integrin molecule VLA-4. This result demonstrates that SLX liposome inhibition of HL-60 and LEC 11 cells is not a stearic effect attributable to binding of liposomes to endothelial cells, but supports the conclusion that SLX liposomes inhibit the adhesion through a direct competition with the ligand binding site of ELAM-1.

EXAMPLE V

Antibodies to SLX Inhibit GMP-140 Mediated Binding on Activated Human Platelets

In this Example the ability of monoclonal antibodies specific for SLX and for the unsialylated Le^x to block the GMP-140 mediated adhesion of HL-60 cells to activated human platelets was determined.

Materials: HL-60 cells are described above and were used as the source of ligand bearing cells. Jurkat cells were used as the non-ligand bearing control. Monoclonal antibodies SH-1, FH-2, SNH-4, and CSLEX-1 are also described above.

Procedure:

1. Blood was drawn from a normal human donor into a syringe containing ACD anticoagulant (dextrose, 2.0 g; sodium citrate 2.49 g; and citric acid 1.25 g; to 100 ml with dH_2O) at a ratio of 6 parts blood to 1 part anticoagulant.

Platelets were isolated by differential centrifugation as follows: Blood was centrifuged at 800 rpm (approx. 90 x g) for 15 min. at room temp. The supernatant was collected and centrifuged at 1200 rpm (approximately 400 x g) for 6 min. The supernatant was removed and centrifuged at 2000 rpm (1200 x g) for 10 min. to pellet the platelets. The platelet button was washed 2 times with Tyrode-HEPES buffer, pH 6.5 (NaCl 8.0 g; KCl 0.2 g; $NaH_2PO_4 \cdot H_2O$ 0.057 g; $MgCl_2 \cdot 6H_2O$ 0.184 g; $NaHCO_3$ 0.1 g; Dextrose, 1.0 g; and HEPES, 2.383 g; bring to 1 L with DI water, adjust to pH 6.5 with 1N NaOH) followed by one wash in PBS. Platelets were suspended to a concentration of 10^8/ml in PBS.

2. Approximately 20 min. before the platelets were finally resuspended, 48 well plates were coated with 0.1% gelatin and incubated to 15 min. at 37°C. Excess gelatin was removed by pipette immediately fore the addition of the platelet suspension. Platelets were activated by the addition of 0.25 units of thrombin/ml (Sigma T-6759) of platelet

suspension. Platelets were allowed to stand at room temperature for 20 min.

3. To prepare bound, activated platelets, 300 µl of the platelet suspension was added to each well of the gel coated plate. The plate was incubated at 37°C to 15 min., then spun at 800 rpm (90 xg) for 2 min. The unbound platelets were removed by washing the plate 3 times with PBS.

4. Since platelets possess highly reactive Fc receptors, to prevent uptake of any aggregated IgG from the antibody preparation, the platelet Fc receptors were blocked as follows: Purified mouse IgG W6/32 (IgG_{2a}) at 27 mg/ml was aggregated by heating at 63°C for 5 min. 300 µl of the heated preparation at 20 µg/ml in PBS was added to each well of the platelet-coated plate. The plate was incubated at 37°C for 15 min. then washed with PBS.

5. HL-60 and Jurkat cells were harvested and washed in CRPMI. A viable cell count was made using trypan blue, 3 x 10^6 cells of each type were placed in each of 2, 10 ml test tubes and 300 µl of ^{51}Cr (450 µCi) (New England Nuclear) was added to each tube. The tubes were incubated for 1 hr. at 37°C with gentle agitation.

6. Radiolabeled cells were washed 3X in CRPMI and pooled into one tube. They were then centrifuged and resuspended to 4 x 10^6 cells per ml in the same medium.

7. 1.6 ml of each monoclonal antibody culture supernatant, and 1.6 ml of purified CSLEX-1 (15 µg) were added to separate test tubes; control tubes received 1.6 ml of medium.

8. 100 µl of the labeled HL-60 of Jurkat cell suspension (containing 4 x 10^5 cells) was added to each of the tubes which contained monoclonal antibody. They were incubated for 20 min. at 37°C with gentle agitation. Following this

incubation period, 0.3 ml of each cell suspension (containing 7.5×10^4 cells) was added to each well of the previously described assay plate containing bound activated platelets. Each assay was done in triplicate.

9. The assay plate was centrifuged at 90 xg for 2 min. and then incubated for 5 min. at room temp. Unbound cells were removed from the wells of the assay plate by inverting the plate into a radioactive waste receptacle and blotting the plate on towels. The wells were washed X3 by carefully adding 300 µl PBS to each well and inverting and blotting the plate. All of the medium was removed from the wells and 0.3 ml of a solution of 0.125 M Tris, 2% SDS and 10% glycerin was added. The plates were allowed to stand for 15 min. and then 0.6 ml of dH_2O was added to each well.

10. The fluid in each well was resuspended with a pipette and transferred to a glass test tube. The tip was ejected into the tube. The tubes, including those containing the input samples, were counted in a gamma counter. CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. Input CPMs were determined by counting a 0.3 ml aliquot of each cell suspension described in step 8.

The results, shown in Fig. 6, indicate that the monoclonal antibodies SNH-4 and CSLEX-1 specific for SLX blocked the binding of HL-60 cells to GMP-140 on activated platelets. The monoclonal antibodies specific for Le^x (FH2 and SH-1) also blocked this binding but to a lesser extent. This Example suggests that both SLX and Le^x may be ligands for GMP-140, but that the SLX structure may be of a higher affinity for GMP-140 than the Le^x structure.

EXAMPLE VI

Liposomes of Glycosphingolipids Block Binding of SLX Cells to Activated Platelets

This Example demonstrates the ability of the liposomes which contain SLX or SLX mimetics to block the binding of SLX-expressing HL-60 cells and PMNs to platelets

which have been stimulated to express GMP-140 by treatment with Thrombin. The assays generally followed the protocol described in Larsen et al., Cell 63: 467-474 (1990), which is incorporated herein by reference.

Materials:

Glycosphingolipids were prepared as described in Example IV. The platelets were prepared as described in Example V, except that blocking of Fc receptors was not performed. HL60 cells were prepared as described above.

PMNs were prepared from 50 ml of whole blood drawn from volunteer donors into heparinized vacutainer tubes, which were inverted to mix the blood. All steps were performed at 22-24 degrees C. Each 25 ml of blood was layered over 15 ml of Mono-Poly Resolving Medium (Flow Labs). The tubes were centrifuged at 800xg for 25 min followed by 1300xg for a further 25 min. The PMN layer was removed and placed in a clean 50cc centrifuge tube. Thirty ml of Hanks Balanced Salt Solution (Gibco) containing 20mM HEPES (Gibco) and 0.2% glucose (Fisher) was added to each tube, which were then centrifuged at 1900xg for 3 min. The PMNs were washed 3X in the same buffer by centrifugation at 1900xg for 3 min. PMNs were counted using a hemacytometer and resuspended to 2 $x10^6$/ml and held at room temperature until use.

Procedure

1. 20 ul of preparation of activated platelets were placed in each of 28 1.5 ml eppendorf tubes (14 duplicate samples).

2. 20 ul of the diluted liposomes at 10 ug, 5 ug or 2 ug, or of the control buffers, were added to the appropriate tube of each duplicate.

3. The platelets were incubated with the liposome preparations for 20 min. at room temp.

4. Neutrophils or HL-60 cells at 2 x 10^6 cells/ml were each added to one set of liposome treated platelets. 20 ul of cell suspension were added to each tube.

5. The tubes were mixed and allowed to stand at room temperature for 20 min. Then they were applied to a

hemacytometer and the cells were scored as positive (2 or more platelets attached/cell) or negative (less than 2 platelets attached/cell).

As shown in Fig. 7, liposomes containing selected glycolipids having terminal sequences which contained SLX (S-diLE^X, Table 1) dramatically inhibited adhesion of HL-60 cells to activated platelets. Liposomes containing glycolipids with Le^X (di-Le^X) or other related carbohydrate structures (Table 1) exhibited minimal inhibition that was not dependent on the structure of the carbohydrate group. Similar results were obtained with PMN cell adhesion (Fig. 8). These experiments show that biosynthetically produced SLX or similar SLX mimetic compounds when formulated into liposomes compositions can serve as therapeutic compounds for, e.g., the reduction of leukocyte binding to platelets in inflammatory sites.

EXAMPLE VII

Oligosaccharide containing SLX blocks binding of Neutrophils to platelets

In this example the ability of a minimal tetra-saccharide SLX to inhibit GMP-140 adhesion was compared to that of penta- and hexasaccharides containing SLX. Briefly, platelets and neutrophils were isolated by the methods described above. Platelets were activated with thrombin and then incubated with dilutions of various oligosaccharides. Neutrophils were added and the effect of the saccharides on the adhesion of neutrophils to activated platelets was determined. The oligosaccharides used were as follows: SLX(hexa), NeuAcα2,3Galβ1,4 (Fucα1,3)GlcNacβ1,3 Galβ1,4Glc-O-$CH_2CH_2SiMe_3$ (the generous gift of Professor Hasegawa, Gifu University, Japan), SLX(penta) NeuAcα2,3Galβ1,4(Fucα1,3)GlcNacβ1,3Galβ, and SLX(tetra), NeuAcα2,3Galβ1,4 (Fucα1,3)GlcNAc.

Procedure

1. Platelets were isolated as described above and were activated ($2x10^8$/ml) by incubation for 20 min at room temperature with thrombin at a final concentration of 0.25U/ml.

2. Neutrophils were isolated by layering heparinized blood over Mono-Poly Resolving Medium (Ficoll-Hypaque-Flow Laboratories), followed by centrifugation for 25 min at 2000rpm and then, a further 25 min at 2500rpm as described above.

3. For the assay, 20 μl of the platelet suspension ($2x10^8$/ml) was placed in an Eppendorf centrifuge tube. An equal volume of the oligosaccharide preparations at concentrations from 200 μg/ml to 0.3 μg/ml, or of glycolipid-liposome preparations (prepared as described, above), at concentrations from 2 μg/ml to 0.25 μg/ml, was added and the tubes were allowed to stand at room temperature for 20 min. Twenty μl of the neutrophil preparation ($2x10^6$/ml) was then added and the tubes were allowed to stand for a further 20 min at room temperature.

4. Adhesion of activated platelets to the neutrophils was assessed microscopically. One hundred neutrophils were evaluated. They were scored as positive if 2 or more platelets were attached and negative if less than 2 platelets were bound. The percent of cells with 2 or more bound platelets was calculated.

The mean of the results of three identical experiments are shown in Table 2.

TABLE 2

OLIGOSACCHARIDE	AMOUNT REQUIRED FOR 50% INHIBITION (μM)
SLX (hexa)	1.8
SLX (penta)	2.2
SLX(tetra)	54.0
Le^x	43.0

As indicated in Table 2 above, approximately 20 times more of the SLX-tetrasaccharide is required for 50% inhibition of GMP-140 mediated binding of neutrophils to thrombin activated platelets than of the SLX-hexasaccharide. The amount of the tetrasaccharide required is approximately that needed for a similar degree of inhibition when the non-sialylated Le^x was used. The pentasaccharide gives 50% inhibition at concentrations similar to those required for 50% inhibition by the hexasaccharide which indicates that the minimal structure for maximum inhibition is closer to a pentasaccharide.

EXAMPLE VIII

Blocking adhesion using variant SLX structures

This example describes experiments testing various glycolipid structures on liposomes. In particular, SY2, a sialylated polysaccharide in which the fucose instead of being attached to the ultimate GlcNAc as in SLX, is attached to the penultimate GLcNAc was tested. Platelets and neutrophils were isolated by the methods described above. Platelets were activated with thrombin and then incubated with dilutions of various glycolipids embedded in liposomes prepared as described above. Neutrophils were added and the effect of the glycolipids on the adhesion of neutrophils to activated platelets was determined.

Structures of the various glycolipids examined are as follows: **SDiY2**, NeuGcα2,3Galβ1,4(Fucα1,3)GlcNacβ1,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; **SLX**, NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; **SY2**, NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; **SH**, NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; **SPG**, NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer.

The results of two identical experiments are shown in Table 3.

Table 3

GLYCOLIPID	AMOUNT REQUIRED FOR 50% INHIBITION (μM)
SY2 (Exp 1)	0.325
SY2 (Exp 2)	0.345
SLX (hexa)	0.30
SdiY2	0.36
SPG	No Inh.
SH	No Inh.

These results show that SY2 inhibited GMP-140 mediated adhesion of neutrophils to thrombin activated platelets equally as well as did SLX and SDiY2.

EXAMPLE IX

Blocking adhesion using further variants of SLX

The example demonstrates that the affinity of sialylated Le^x(SLX) for GMP-140 is the same whether the terminal sialic acid is in the form N-Acetyl neuraminate (NeuAc) or N-Glycol neuraminate (NeuGc). All materials were prepared as described above. Platelets and neutrophils were isolated by the methods described. Platelets were activated with thrombin and then incubated with dilutions of various glycolipids contained in liposomes. Neutrophils were added and the effect of the glycolipids on the adhesion of neutrophils to activated platelets was determined.

The results of an experiment in which synthetic SLX(NeuAc) and a preparation of SLX prepared by enzymatic fucosylation of sialylparagloboside purified from bovine erythrocytes SLX(NeuGc), were directly compared are shown in Table 4.

Table 4

GLYCOLIPID	SOURCE	AMOUNT REQUIRED FOR 50% INHIBITION (μM)
SLX (NeuGc)	(Bovine Erythrocytes)	0.74
SLX (NeuAc)	(Synthetic)	0.67

These results show that SLX-hexasaccharide inhibited GMP-140 mediated adhesion of neutrophils to thrombin activated platelets equally well whether the sialic acid was NeuAc or NeuGc. This result indicates that either the N-acetyl or N-glycollyl derivative of sialic acid are recognized by GMP-140. Similar results have been obtained with ELAM-1.

Various glycolipids were also tested in the same assay. Structures of the glycolipids tested are as follows: **SLX(hexa)**, NeuGcα2,3Galβ1,4(Fucα1,3)GlcNacβ1,3Galβ1,4 4Glcβ1,1Ceramide; **α2,3 SLX cer,** NeuAcα2,3Galβ1,4(Fucα1,3) GlcNAcβ1,3Galβ1,4Glcβ1,1Ceramide; **α2,6 SLX cer,** NeuAcα2,6Galβ1,4(fucα1,3)GlcNacβ1,3Galβ1,4Glcβ1,1Ceramide; **SH,** NeuGcα2,3Galβ1,4 GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Ceramide.

EXAMPLE X

Blocking adhesion using synthetic SLX

This example demonstrates that synthetic SLX binds ELAM-1 and inhibits neutrophil adhesion to activated endothelium. This example also shows that the linkage of the sialic acid affects binding to ELAM-1.

Two synthetic compounds were prepared. One comprised sialic acid in an α2,3 linkage, as in naturally occurring SLX. The second comprised sialic acid in an α2,6 linkage, to examine the importance of the nature of the linkage to receptor binding.

Liposomes were prepared by adding 12 μl of absolute ETOH to each tube, warming briefly in a 50°C water bath and sonicating for 2 min. 238 μl of warm phosphate buffered saline (PBS) was added slowly to each tube while sonicating and sonication was continued for a further 10 min. The final concentration of stock liposomes was 400 μg glycolipids/ml in 5% ETOH/PBS.

Procedure

1. HUVECs, PMNs, and liposomes were prepared as described above.

2. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed two times with RPMI 1640 containing 5 mg/ml bovine serum albumin (BSA).

3. Liposomes stocks were diluted in the HBSS/BSA buffer to make solutions equal to: 40 μg/ml, 30 μg/ml, 15 μg/ml, 7.5 μg/ml, 3.75 μg/ml and 1.87 μg/ml. Similar dilutions were prepared from a control stock consisting of PBS-5% ETOH.

4. The medium was removed from the wells of the assay plate with a pasteur pipette, a few wells at a time.

5. 0.05 ml of each liposome suspension was added to duplicate wells on the stimulated assay plate. Control wells received the liposome buffer containing ethanol HBSS and BSA at the same concentrations as in the liposome containing wells. Control buffer was plated on unstimulated and stimulated HUVEC. Liposome containing samples were added to stimulated wells only.

6. The plates were incubated for 40 min at 37°C and then 50 μl of PMNs were added to the assay wells. The final concentration of cells was $5x10^5$ well in 100 μl.

7. The assay plate was returned to the incubator (5% CO_2, 37 °C) for 8 min.

8. Unbound cells were removed from the wells of the assay plates by systematic resuspension using a p200 multichannel pipette followed by addition and removal of 0.2 ml of medium.

9. All of the medium was removed from the wells and 50 μl of solubilization buffer was added. This consisted of citrate buffer (24.3 ml of 0.1 M Citric acid, 10.5 g/500 ml + 25.7 ml of 0.2 M dibasic sodium phosphate, 14.2 g/500 ml and SQ H_2O to 100 ml) containing 0.1% NO-40 detergent.

10. The plate was incubated on a rotary shaker for 10 min and then 0.05 ml of OPDA solution [8 mg o-phenylene-diamine, Sigma cat# P-1526, 8 μl of 30% H_2O_2 and 10 ml of citrate buffer (as above)] was added to each well. The reaction

was allowed to develop for 15 min and then 25 µl of 4N H_2SO_4 was added to each well to stop the reaction.

11. A reagent bulk was prepared by mixing 100 µl volumes of the solubilization buffer and the OPDA solution with 50 µl of 4N H_2SO_4.

12. 100 µl of supernatant was removed from each of 2 wells and transferred to a flexible ELISA assay plate (Falcon). The plate scanned spectrophotometrically at 492 nM within 30 min.

The results of the two experiments are presented in Table 5, below.

Table 5

	Concentration	(2,6) SLex Mean	(2,3)SLex Mean
1	20 µg/ml	0.663	0.156
2	15 µg/ml	0.636	0.270
3	7.5 µg/ml	0.602	0.359
4	3.75 µg/ml	0.655	0.483
5	1.87 µg/ml	0.690	0.580
6	.47 µg/ml	0.695	0.642
7	0 µg/ml	0.710	0.716

	Concentration	Control +1L-1B Mean	Control -1L-1B Mean
1	20 µg/ml	0.657	0.010
2	15 µg/ml	0.740	0.010
3	7.5 µg/ml	0.658	0.013
4	3.75 µg/ml	0.698	0.009
5	1.87 µg/ml	0.725	0.014
6	.47 µg/ml	0.782	0.018
7	0 µg/ml	0.708	0.016

These results show that liposomes containing synthetic α(2,3)SialylLe^x but not α(2,6)SialylLe^x inhibit neutrophil adhesion to activated endothelium in an ELAM-1 dependent binding assay. Thus, the α2,3 linkage of the sialic acid appears to be necessary for recognition by ELAM-1. In addition, the results show that a synthetically produced oligosaccharide, α(2,3)SialylLe^x, binds to ELAM-1 and blocks binding of neutrophils to activated endothelium. Similar results were found with GMP-140 and are presented in Figure 9. Similar results were found with GMP-140. See Figure 9. This compound or derivatives of this compound therefore constitute potential anti-inflammatory drug candidates.

EXAMPLE XI

Treatment of HL60 Cells with Endo-β-Galactosidase

This example describes experiments to determine whether the internal β-galactose-backbone sugar linkage of sialylated Le^x of HL60 cells was susceptible to cleavage by Endo-β-Galactosidase, an enzyme known to cleave an internal β-galactose linkage in polylactosaminyl structures, but not β-gal when GlaNAc is attached to mannose (core-type structures).

Procedure:

Platelets were isolated and activated with thrombin by the methods described above. Il-1β activated HUVEC were prepared as described above. Cultured HL60 cells were treated with endo-β-galactosidase as described below and the effect of enzyme treatment on the GMP-140 mediated adhesion of HL60 cells to activate platelets was determined.

Enzyme treatment of the HL60 cells was carried out as follows: 12.4 x10^6 cells were washed twice with Hanks Balanced Salt Solution containing 20mM HEPES and 0.2% glucose, followed by a single wash step in normal saline. The endo-β-galactosidase (0.1 Unit, ICN Chemicals, Inc., Irvine, CA) was dissolved in 200μl normal saline and 200μl sodium acetate buffer, pH 6.01. 200μl (containing 0.05U of enzyme) was added to 3 x 10^6 HL60 cells, and 200μl of the acetate buffer was added to a similar number of cells to be used as the buffer control. Both tubes were incubated at 37°C for 60 min. with

gentle shaking. The tubes were then cooled in ice and the cells were washed three times in HBSS containing HEPES and glucose and were then counted and suspended to $2x10^6$/ml.

For the GMP-140 assay, 20μl of Tyrode-HEPES buffer, pH 7.2 was placed in an Eppendorf tube. The same volume of activated platelets ($2x10^8$/ml) and HL60 cells ($2x10^6$/ml) was added and, after mixing, the tubes were allowed to stand at room temperature for 20 min. Adhesion of platelets to the HL60 cells was assessed microscopically as described earlier for adhesion of activated platelets to neutrophils.

For the ELAM-1 assay, enzyme treatment of the HL-60 cells was performed as described above, except that the cells were simultaneously labeled with ^{51}CR as previously described. ELAM-1 mediated adhesion was arrested by incubating 2 x 10^5 treated or untreated cells with IL-1 activated HUVEC for 30 minutes at 4°C and then washing the plate with a pasteur pipette.

The results of these experiments indicated that treatment of HL60 cells with Endo-β-Galactosidase inhibited their ability to bind to (1) thrombin activated platelets by 87.5% and (2) IL-1β activated HUVEC at 4°C by 70%. Thus, the minimal SLX-containing tetrasaccharide ligand for GMP-140 is probably attached to a lactose or polylactosaminyl structure rather than a mannose.

EXAMPLE XII

Fucosylated Polysaccharide blocks binding of Neutrophils to Platelets

In this example the ability of a fucosylated polysaccharide to inhibit GMP-140 mediated adhesion was compared to that of the non-fucosylated polysaccharide, a hexasaccharide SLX and Le^x. Briefly, platelets and neutrophils were isolated by the methods described above. Platelets were activated with thrombin and then incubated with dilutions of various oligosaccharides. Neutrophils were added and the effect of the saccharides on the adhesion of neutrophils to activated platelets was determined. The oligosaccharides used were as follows: Native polysaccharide and its fucosylated derivative (the preparation of both is described, below); SLX

hexasaccharide, LNF III (Le^x) and LNF I (the structures are described above).

The conversion of a polysaccharide which contains the linear core structure of SLX into a polyvalent SLX containing polysaccharide was achieved by enzymatic fucosylation. The native polysaccharide type Ia was obtained from Group B Streptococcus as described by Jennings et al., Biochem. 22 1258-1263 (1983) which is incorporated herein by reference. The appropriate bacterial strains are deposited with the American Type Culture Collection and have Deposit Nos. 12400, 31574, 12401, and 31575.

To prepare the fucosylated polysaccharide, the native type Ia polysaccharide 1 mg. was dissolved in a mixture of 6 μL of 1 M manganese chloride, guanosine 5'-diphosphate β-L-fucose with a radiolabelled tracer (specific activity 1.82 x 10^6 cpm/μmol), 0.9 μmoles in water 90 μL and water 137 μL. To this was added 100 μL solution of 3/4 fucosyl transferase isolated from human milk as previously described by Prieels et al., J.Biol.Chem. 256 10456-10463 (1981) which is incorporated herein by reference.

The reaction mixture was concentrated against a membrane (100K cut off) several times with water and the retentate lyophylized to give a powder. This solid was dissolved in water and passed through a weak cation exchange column to remove any remaining protein. The radioactive fractions containing the fucolsylated polysaccharide were collected and lyophilized. Approximately fifty of the available side chains were fucosylated as measured by the incorporation of the radiolabel.

Procedure:

Platelets were isolated as described above and were activated ($2x10^8$/ml) by incubation for 20 min at room temperature with thrombin at a final concentration of 0.25U/ml.

Neutrophils were isolated by layering heparinized blood over Mono-Poly Resolving Medium (Ficoll-Hypaque, Flow Laboratories), followed by centrifugation for 25 min at 2000rpm and then, a further 25 min at 2500rpm as described above.

For the assay, 20 μl of the platelet suspension ($2x10^8$/ml) was placed in an Eppendorf centrifuge tube. An equal volume of the oligosaccharide preparations at concentrations from 500μg/ml to 2.0μg/ml was added and the tubes were allowed to stand at room temperature for 20 min. Twenty μl of the neutrophil preparation ($2x10^6$/ml) was then added and the tubes were allowed to stand for a further 20 min at room temperature.

Adhesion of activated platelets to the neutrophils was assessed microscopically. One hundred neutrophils were evaluated. They were scored as positive if 2 or more platelets were attached and negative if less than 2 platelets were bound. The percent of cells with 2 or more bound platelets was calculated.

As shown in Table 6, the fucosylated polysaccharide very efficiently inhibited GMP-140 mediated binding of neutrophils to thrombin activated platelets- 50% inhibition was achieved with less than 1μg/ml. This compared to 20μg/ml which was required of the native polysaccharide and 8μg/ml of the SLX hexasaccharide for a similar degree of inhibition.

TABLE 6

OLIGOSACCHARIDE	AMOUNT REQUIRED FOR 50% INHIBITION (μM)
Native Polysaccharide	15
Fucosylated Polysaccharide	0.7
SLe^x Hexasaccharide	8
LNF III (Le^x)	35
LNF I	No Inhibition

EXAMPLE XIII

Protection of Rats from Endotoxic Shock (Lethality) by a Monoclonal Antibody to LAM-2

This example demonstrates the efficacy of mAb P6E2 (a murine IgG3k, functional anti-human ELAM-1 mAb, described in copending application U.S.S.N. 07/645,878, which is incorporated herein by reference) in an animal model of lipopolysaccharide-induced death. A rat system was chosen because P6E2 has been shown to cross-react with the rat equivalent to ELAM-1.

Materials and Methods

LPS from E. coli 0111:B4 (Sigma) was prepared fresh from a single lot one day prior to use by dissolving in sterile, pyrogen-free saline at a concentration of 5 mg/ml. The solution was sonicated on ice for 30 seconds using a Tekmark Sonic disrupter. Just prior to use, the material was sonicated a second time for 30 seconds.

Female Lewis rats' weighing 200 g (+- 10g) were purchased from Charles River Breeding Labs and held for at least 7 days after receipt (for adaptation). Groups (10 animals) were used, unless otherwise noted. All reagents were injected parenterally via tail vein. As negative controls, animals received either sterile, LPS-free saline, or a murine lgG3k myeloma protein (J606, low pyrogen - < 2 ng/mg protein).

Results

The P6E2 dose/schedule protocols were arrived at empirically from the pharmacokinetic data we obtained with P6E2 prophylactically administered to rats. A "minimal" LD_{100} dose was empirically determined to be 7.5 mg/kg for these rats.

In one experiment, rats were treated with 10 mg/kg one hour before the LPS challenge. A boost was administered 3 hours after the challenge. 4/10 treated animals survived the LPS challenge. In contrast, all 10 (saline-injected) controls died. At the twenty-four hour observation period, the

survivors showed few of the clinical signs characteristic of LPS-treated animals.

We next tried doses of P6E2 which were (1) 2-fold higher and (2) an order of magnitude lower than the 10 mg/kg dose used in the first experiment. The results show that P6E2 had a significant effect: 80% of the animals survived at the 10 mg/kg dose (Figure 10). Note that one animal survived in the saline control group - suggesting that this group of animals was not "hit" as hard.

Another study was performed to demonstrate the therapeutic value of P6E2. Animals received the 10 mg/kg iv bolus dose of P6E2 at 1 hour before or 2, 4 or 6 hours after the LPS challenge.

Once again, 1 of 10 animals survived in the saline-treated group, as well as in a group treated with 10 mg/kg J606 myeloma protein at T=-60 minutes (Figure 11). P6E2 had a protective effect even when administered 2 or 4 hours after LPS.

Conclusion

The protection seen with Cytel mAb P6E2 demonstrates of the importance of ELAM-1 in an animal model of a lethal disease.

EXAMPLE XIV

Rolling of SLex Liposomes on 1L-1 Activated Rabbit Endothelial Cells.

Purpose

This example demonstrates that sialylated Lewis x (SLX) and sialoparagloboside (SPG) isolated from biological sources and incorporated into liposomes will "roll" along activated rabbit venules.

"Rolling" is an early intercellular interaction between leukocytes and the endothelial cell wall. A leukocyte will "roll" on endothelial cells. The leukocyte will then either (1) be released back into circulation, or (2) adhere to the endothelial cell, and begin the early events that culminate in inflammation. Selectins have been implicated in the cell-cell

interactions of "rolling." von Adrian, et al., Proc. Nat'l Acad. Sci., ____:____ (1991).

Materials

Liposomes containing the glycosphingolipids were formed as follows: 50 ug of glycolipid was added to 150 ug phosphatidylcholine (Sigma, egg yolk), 250 ug cholesterol (Sigma) and 1 mM carboxyfluorescein (Sigma) in chloroform:methanol (2:1) and the whole solution evaporated to dryness in glass screw cap tubes. Liposomes were prepared by adding 12.5 ul of absolute ethanol to each tube, warming briefly in a water bath and sonicating for 2 minutes 238 ul of warm PBS was added slowly to each tube while sonicating and sonication was continued for a total of 10 minutes. The liposomes were brought to 1 ml with PBS and centrifuged at 14,000 rpm for 2 minutes to remove excess EtOH and carboxyflourescein. The supernatant was discarded and the liposomes resuspended in 1 ml PBS, counted on a hemacytometer and adjusted to $5x10^6$ liposomes per ml. One ml is injected into the rabbit, activated 4 hours earlier with IL-1.

Rolling was observed using intravital microscopy as described by von Adrian et al. IL-1 activated rabbit mesentery was exteriorized and spread on a 37°C heated glass window over a microscopic stage. The mesentery was bathed with 36.5°C saline solution equilibrated with 5% CO_2 in N_2. Liposome-endothelial interactions were observed with an intravital microscope with a 50X salt water immersion objective. Liposomes were visualized by exposure to fluorescent light. The liposomes in a segment of selected mesentery venule (20-40μm in diameter) were counted for one minute.

Results

Liposome Rolling on IL-1 Activated Rabbit Cells

material injected	rolling
human neutrophils	4 (maximum amount of rolling)
SLex liposomes	2
SPG liposomes	0 (no rolling)

Conclusion

In addition to LAM-1 (von Andrian, et al.), ELAM-1 and it's ligand appear to be involved in the process of leukocyte margination or "rolling".

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound comprising a moiety which selectively binds a selectin receptor, the moiety having the formula:

R_1—Galβ1,4(Fucα1,3)GlcNAc—$(R_2)_a$

wherein R_1 is an oligosaccharide or R_3—R_4—C(CO_2H)-,

wherein R_3 and R_4 are the same or different and are —H, — C1-C8 alkyl, — hydroxyl C1-C8 alkyl, —aryl C1-C8 alkyl, or —alkoxy C1-C8 alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

2. A composition of claim 1, wherein the compound is a biomolecule.

3. A composition of claim 1, wherein the biomolecule is an oligosaccharide, an oligopeptide, a protein, or a lipid.

4. A composition of claim 1, wherein R_3 and R_4 are connected to form a 4-8 membered ring.

5. A composition of claim 4, wherein the 4-8 membered ring is a monosaccharide.

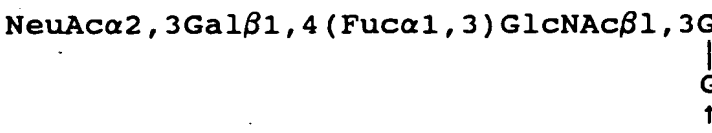
6. A composition of claim 5, wherein the monosaccharide is a sialic acid.

7. A composition of claim 6, wherein the sialic acid is NeuAcα2,3 or NeuGcα2,3.

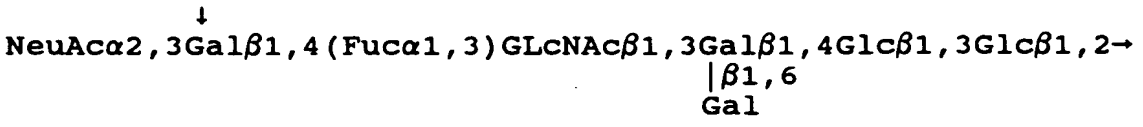
8. A composition of claim 1, wherein the oligosaccharide is NeuAcα2,3Galβ1,4GlcNAcβ1,3 or NeuGcα2,3Galβ1,4GlcNAcβ1,3.

9. A composition of claim 1, wherein the compound is a polysaccharide.

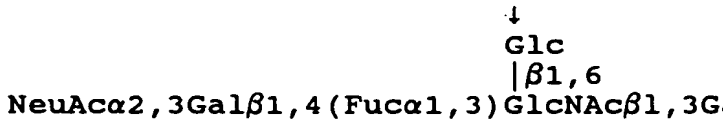
10. A composition of claim 9, wherein the polysaccharide comprises a repeat unit having the formula:



11. A composition of claim 9, wherein the polysaccharide comprises a repeat unit having the formula:



12. A composition of claim 9, wherein the polysaccharide comprises a repeat unit having the formula:



13. A composition of claim 9, wherein the polysaccharide is a fucosylated type Ia polysaccharide of Group B streptococcus.

14. A composition of claim 9, wherein the polysaccharide is a type II or type III polysaccharide of Group B streptococcus.

15. A composition of claim 9, wherein the polysaccharide has molecular weight between about 5,000 and 300,000 daltons.

16. A composition of claim 9, wherein the polysaccharide comprises between about 5 and about 200 fucosylated repeat units.

17. A composition of claim 16, wherein the polysaccharide comprises between about 25 and about 100 fucosylated repeat units.

18. A composition of claim 1, wherein the biomolecule is a sphingolipid.

19. A composition of claim 18, wherein the biomolecule is a ganglioside.

20. A composition of claim 1, wherein the selectin receptor is expressed on a vascular endothelial cell or a platelet.

21. A composition of claim 20, wherein the selectin receptor is ELAM-1 or GMP-140.

22. A pharmaceutical composition which comprises a pharmaceutically acceptable carrier and a liposome having a compound which selectively binds a selectin receptor.

23. A composition of claim 22, wherein the liposome encapsulates an anti-inflammatory chemotherapeutic agent.

24. A composition of claim 23, wherein the anti-inflammatory agent is cyclosporin A, indomethacin, naproxen, FK-506, or mycophenolic acid.

25. A composition of claim 22, wherein the compound has the formula

R_1—Galβ1,4(Fucα1,3)GlcNAc—$(R_2)_a$—X

wherein R_1 is selected from the group consisting of NeuAcα2,3, NeuGcα2,3, NeuAcα2,3Galβ1,4GlcNAcβ1,3, and NeuGcα2,3Galβ1,4GlcNAcβ1,3;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1; and

wherein X is a protein or lipid.

26. A composition of claim 25, wherein X is a glycoprotein having a molecular weight between 40,000 and about 250,000 daltons.

27. A composition of claim 25, wherein X is a glycolipid having a molecular weight between about 600 and about 4,000 daltons.

28. A composition of claim 22, wherein the compound comprises a moiety having the formula:

NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc–$(R)_a$–,

NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAc–$(R)_a$–, and

NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAc–$(R)_a$–;

wherein R is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

29. A composition of claim 22, wherein the selectin receptor is expressed on a vascular endothelial cell or a platelet.

30. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound which selectively binds a selectin receptor, the compound having the formula:

R_1–Galβ1,4(Fucα1,3)GlcNAc–$(R_2)_a$–X

wherein R_1 is an oligosaccharide or R_3–R_4–$C(CO_2H)$-,

wherein R_3 and R_4 are the same or different and are –H, –lower alkyl (C1-C8), –hydroxyl lower alkyl, –aryl alkyl, or –alkoxy alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1; and

wherein X is selected from the group consisting of –H, –OH, $-NH_3$, $-NHR_3$, $-NR_3R_4$, $-OR_3$, –OAryl, –OAlkylAryl, –OArylAlkyl, $-R_3$, –Aryl, –ArylAlkyl, and –AlkylAryl, wherein R_3 and R_4 are the same or different and are C_1-C_{20} alkyl.

31. A composition of claim 30, wherein the compound has the formula:

NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc–$(R)_a$,

NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAc–$(R)_a$, and

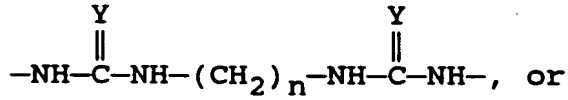
NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAc–$(R)_a$;
wherein R is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

32. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound having two or more repeat units capable of selectively binding a selectin receptor, the repeat units comprising a selectin-binding moiety and being linked by a linker moiety, each repeat unit having the formula:

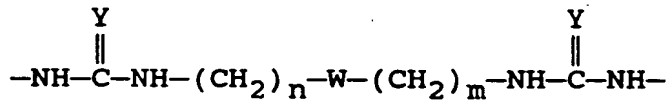
R_1–Galβ1,4(Fucα1,3)GlcNAc–$(R_2)_a$

wherein R_1 is an oligosaccharide or R_3–R_4–$C(CO_2H)$–,
wherein R_3 and R_4 are the same or different and are –H, – C1-C8 alkyl, – hydroxyl C1-C8 alkyl, –aryl C1-C8 alkyl, or –alkoxy C1-C8 alkyl;
wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1; and
wherein X is the linker moiety.

33. A composition of claim 32, wherein the linker moiety has the formula:

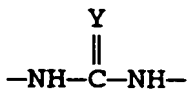


or



wherein, n and m are the same or different and are integers from 2 to 12; Y is O or S; and W is O, S, or NH.

34. A composition of claim 32, wherein the linker moiety is 5- to 14-membered ring having two substituents, each substituent having the formula



wherein, Y is O or S; and
the substituents being in a cis- or trans-relationship.

35. A composition of claim 34, wherein the substituents are in a 1,2 to 1,(p/2)+1 arrangement, wherein p is an integer from 5 to 14 and corresponds to the size of the ring.

36. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a heterocyclic compound having two nitrogen atoms and two selectin-binding moieties, each moiety being linked to one of the nitrogen atoms and having the formula:

R_1—Galβ1,4(Fucα1,3)GlcNAc—$(R_2)_a$

wherein R_1 is an oligosaccharide or R_3—R_4—C(CO_2H)-,

wherein R_3 and R_4 are the same or different and are —H, - C1-C8 alkyl, - hydroxyl C1-C8 alkyl, —aryl C1-C8 alkyl, or —alkoxy C1-C8 alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

37. A composition of claim 36, wherein the heterocyclic compound is piperazine or homopiperazine.

38. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an amino acid linked to a selectin-binding oligosaccharide moiety selected from the group consisting of

NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc—$(R)_a$—,

NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAc—$(R)_a$—, and

NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAc—$(R)_a$—;

wherein R is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

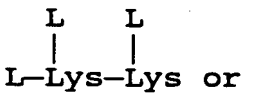
39. A composition of claim 38, wherein the amino acid is lysine, homolysine, ornithine, diaminobutyric acid, asparagine or diaminopropionic acid.

40. A composition of claim 38, wherein the amino acid is incorporated in an oligopeptide.

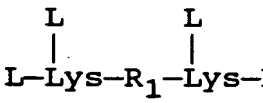
41. A composition of claim 40, wherein the oligopeptide comprises one or more of the following: lysine, homolysine, ornithine, diaminobutyric acid, asparagine or diaminopropionic acid.

42. A composition of claim 41, wherein the oligopeptide further comprises one or more of the following: alanine, tyrosine or radioiodinated tyrosine.

43. A composition of claim 40, wherein the oligopeptide comprises, in a direction from the N-terminus to the C-terminus,



or



wherein R_1 and R_2 are the same or different and are any amino acid residue and L is the oligosaccharide moiety.

44. A pharmaceutical composition for treating an inflammatory condition, the composition comprising a pharmaceutically acceptable carrier and an immunoglobulin capable of selectively binding an oligosaccharide ligand recognized by a selectin cell surface receptor, the immunoglobulin being present in an amount sufficient to treat the condition.

45. A composition of claim 44, wherein the ligand is selected from the group consisting of

NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc–$(R)_a$–,

NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAc–$(R)_a$–, and

NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAc–$(R)_a$–;
wherein R is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

46. A composition of claim 44, wherein the oligosaccharide moiety is expressed by a leukocyte.

47. A composition of claim 44, wherein the selectin receptor is expressed by a vascular endothelial cell or a platelet.

48. A composition of claim 44, wherein the selectin receptor is ELAM-1 or GMP-140.

49. A composition of claim 44, wherein the composition is in unit dosage form.

50. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound comprising a moiety which selectively binds a selectin receptor, the moiety having the formula:

R_1–Galβ1,4(R_2)GlcNac–$(R_3)_a$–,

wherein R_1 is NeuAcα2,3, NeuGcα2,3, NeuAcα2,3, Galβ1,4GlcNAcβ1,3, or NeuGcα2,3Galβ1,4GlcNacβ1,3;

wherein R_2 is Fucα1,3, Araα1,3, (R,S)-5-alkyl-Araα1,3 and (R,S)-5-aryl-Araα1,3; and

wherein R_3 is 1,3βGal, 1,2αMan, or 1,6αGalNAc and a is 0 or 1.

51. A composition of claim 51, wherein the compound is a biomolecule.

52. A composition of claim 51, wherein the moiety binds a selectin receptor expressed on a vascular endothelial cell or a platelet.

53. A composition of claim 51, wherein the selectin receptor is ELAM-1 or GMP-140.

54. A method for inhibiting selectin-mediated intercellular adhesion in a patient, the method comprising administering to the patient a therapeutically effective dose of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound which selectively binds a selectin receptor.

55. A method of claim 55, wherein the compound comprises a moiety which selectively binds a selectin receptor, the moiety having the formula:

R_1—Galβ1,4(Fucα1,3)GlcNAc—$(R_2)_a$

wherein R_1 is an oligosaccharide or R_3—R_4—C(CO_2H)-,

wherein R_3 and R_4 are the same or different and are —H, —lower alkyl (C1-C8), —hydroxyl lower alkyl, —aryl alkyl, or —alkoxy alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

56. A method of claim 55, wherein the compound is a biomolecule.

57. A method of claim 55, wherein the intercellular adhesion is associated with an inflammatory condition.

58. A method of claim 58, wherein the inflammatory condition is septic shock.

59. A method of claim 58, wherein the inflammatory condition is acute respiratory distress syndrome or wound associated sepsis.

60. A method of claim 55, wherein the intercellular adhesion is associated with metastasis.

61. A method of claim 55, wherein the selectin receptor mediates adhesion of a leukocyte, monocyte or neutrophil to an endothelial cell.

62. A method of claim 55, wherein the selectin receptor is ELAM-1 or GMP-140.

63. A method of claim 55, wherein the compound is embedded in a liposome.

64. A method of claim 55, the compound is a polysaccharide.

65. A method of treating an inflammatory disease process mediated by a selectin receptor in a patient, the method comprising administering to the patient a therapeutically effective dose of a compound which selectively binds the receptor, the compound having the formula:

R_1—Galβ1,4(Fucα1,3)GlcNAc—$(R_2)_a$—X

wherein R_1 is an oligosaccharide or R_3—R_4—C(CO_2H)-,

wherein R_3 and R_4 are the same or different and are —H, —lower alkyl (C1-C8), —hydroxyl lower alkyl, —aryl alkyl, or —alkoxy alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1; and

wherein X is a biomolecule.

66. A composition of claim 66, wherein X is an oligosaccharide, an oligopeptide, a protein, or a lipid.

67. A method of claim 66, wherein the selectin receptor is ELAM-1 or GMP-140.

68. A method of assaying a test compound for the ability to inhibit selectin-mediated cellular adhesion, the method comprising the steps of:

contacting the test compound with a selectin receptor and an isolated selectin-binding agent; and

detecting the ability of the test compound to inhibit binding between the receptor and the agent.

69. A method of claim 69, wherein the agent comprises an SLX moiety, or an SLX mimetic.

70. A method of claim 69, wherein the receptor or the agent are immobilized on a solid surface.

71. A method of claim 69, wherein the test compound is an oligosaccharide or a glycoconjugate.

72. A pharmaceutical composition comprising a compound capable of blocking selectin-mediated cellular adhesion identified by the method of claim 69.

73. A method for preparing a compound comprising an oligosaccharide moiety capable of selectively binding a selectin receptor, the method comprising fucosylating a polysaccharide comprising a sequence having the formula:

R—Galβ1,4GlcNAc—

wherein R is a sialic acid.

74. A method of claim 74, wherein the step of fucosylating is carried out using a α1,3 fucosyltransferase.

75. A method of claim 74, wherein the polysaccharide is a type Ia polysaccharide of Group B streptococcus.

76. A method of claim 74, wherein the polysaccharide is a type II or type III polysaccharide of Group B streptococcus.

77. A method for preparing a compound comprising a plurality of moieties capable of selectively binding a selectin receptor, the method comprising linking the moieties together using a linker moiety.

78. A method of claim 78, wherein the moieties are selected from the group consisting of

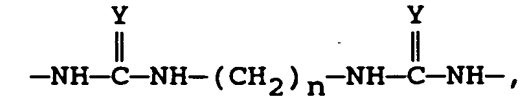
R_1—Galβ1,4(Fucα1,3)GlcNAc—$(R_2)_a$

wherein R_1 is an oligosaccharide or $R_3-R_4-C(CO_2H)-$,

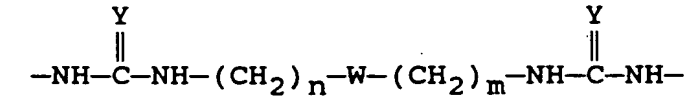
wherein R_3 and R_4 are the same or different and are –H, – C1-C8 alkyl, – hydroxyl C1-C8 alkyl, –aryl C1-C8 alkyl, or –alkoxy C1-C8 alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

79. A method of claim 78, wherein the linker moiety has the formula:

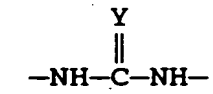


or



wherein, n and m are the same or different and are integers from 2 to 12; Y is O or S; and W is O, S, or NH.

80. A method of claim 78, wherein the linker moiety is 5- to 14-membered ring having two substituents, each substituent having the formula



wherein, Y is O or S; and

the substituents being in a cis- or trans-relationship.

81. A method of claim 78, wherein the substituents are in a 1,2 to 1,(p/2)+1 arrangement, wherein p is an integer from 5 to 14 and corresponds to the size of the ring.

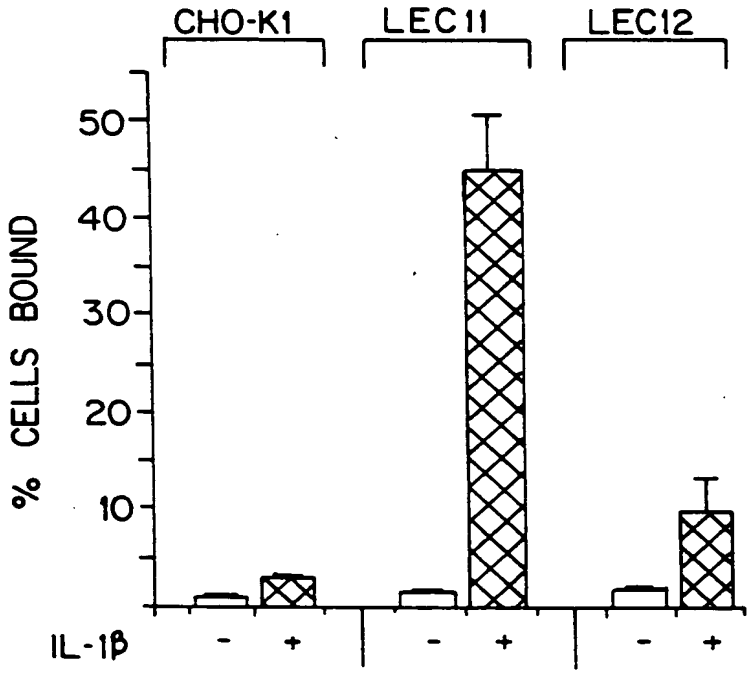


FIG. 1.

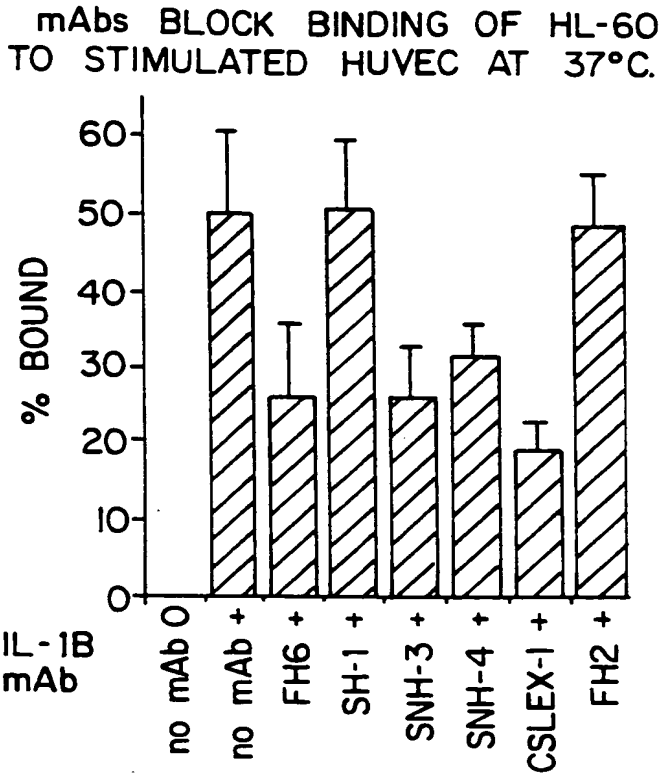

mAbs BLOCK BINDING OF HL-60
TO STIMULATED HUVEC AT 37°C.
% BOUND
60
50
40
30
20
10
0
IL-1B
mAb
no mAb
no mAb
FH6
SH-1
SNH-3
SNH-4
CSLEX-1
FH2
0
+
+
+
+
+
+
+
+

FIG. 2A.

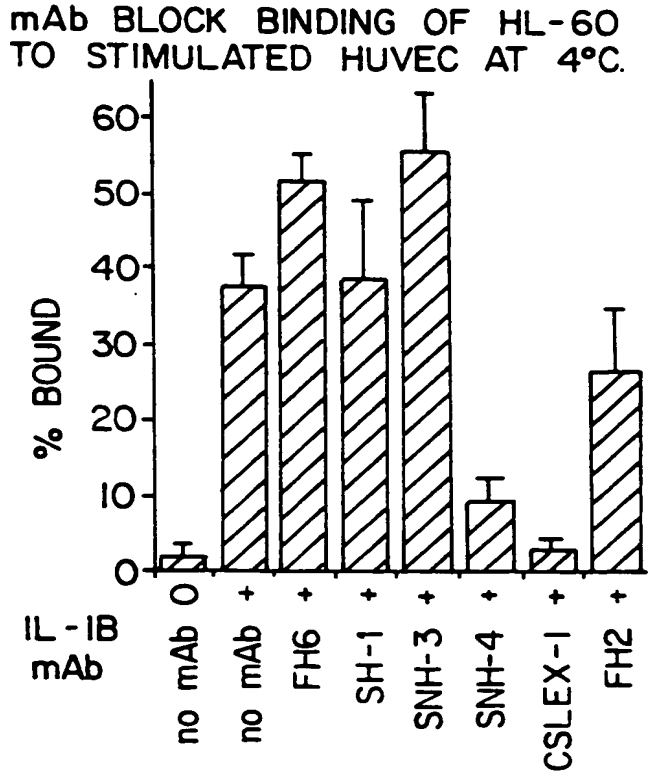

mAb BLOCK BINDING OF HL-60
TO STIMULATED HUVEC AT 4°C.
% BOUND
60
50
40
30
20
10
0
IL-1B
mAb
no mAb
no mAb
FH6
SH-1
SNH-3
SNH-4
CSLEX-1
FH2
0
+
+
+
+
+
+
+
+

FIG. 2B.

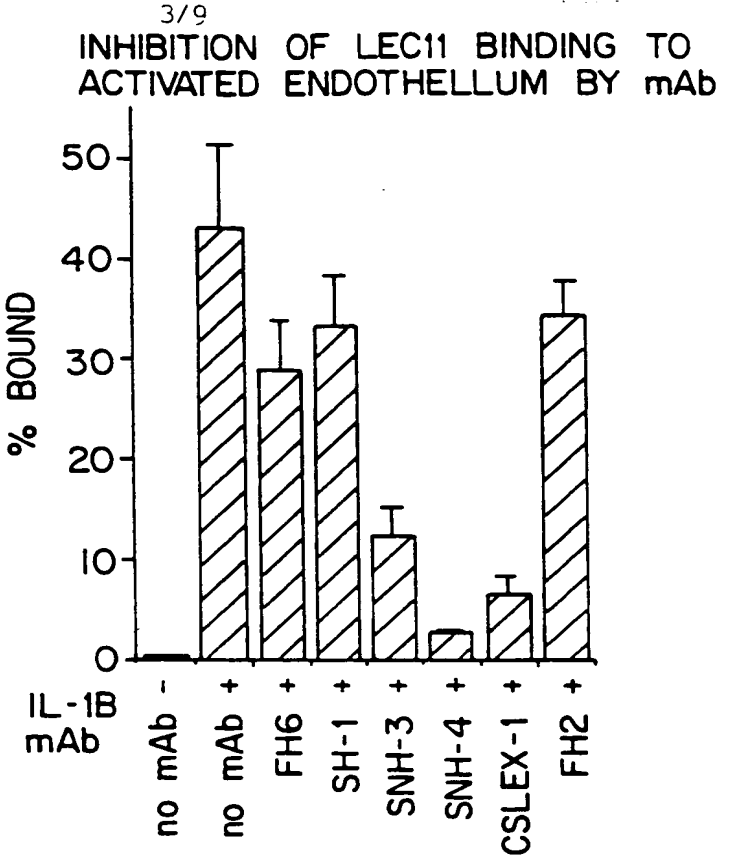

INHIBITION OF LEC11 BINDING TO ACTIVATED ENDOTHELLUM BY mAb
% BOUND
50
40
30
20
10
0
IL-1B -
mAb
no mAb
+
no mAb
+
FH6
+
SH-1
+
SNH-3
+
SNH-4
+
CSLEX-1
+
FH2

FIG. 3A.

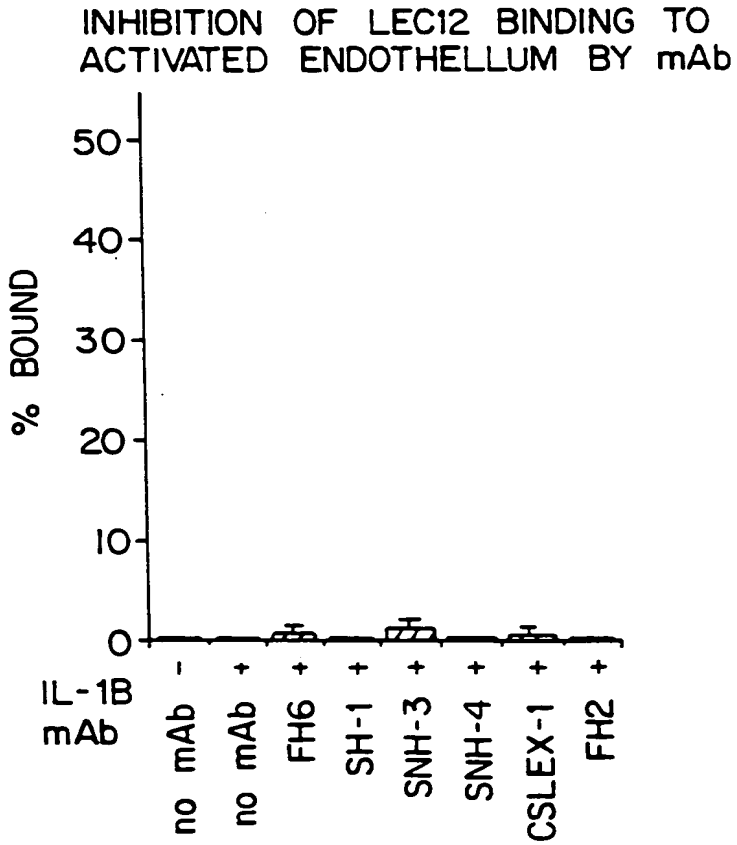

INHIBITION OF LEC12 BINDING TO ACTIVATED ENDOTHELLUM BY mAb
% BOUND
50
40
30
20
10
0
IL-1B -
mAb
no mAb
+
no mAb
+
FH6
+
SH-1
+
SNH-3
+
SNH-4
+
CSLEX-1
+
FH2

FIG. 3B.

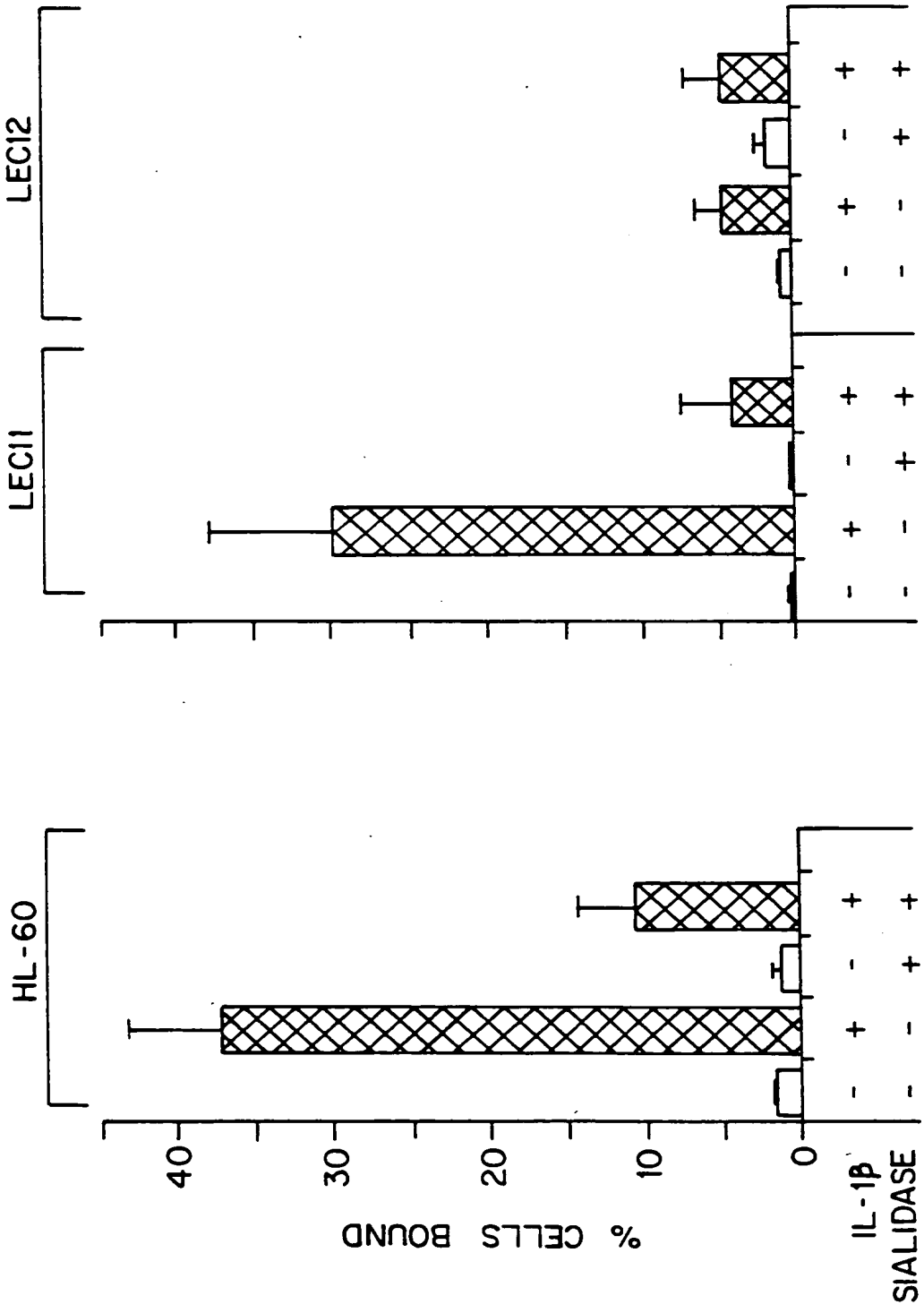


FIG. 4.

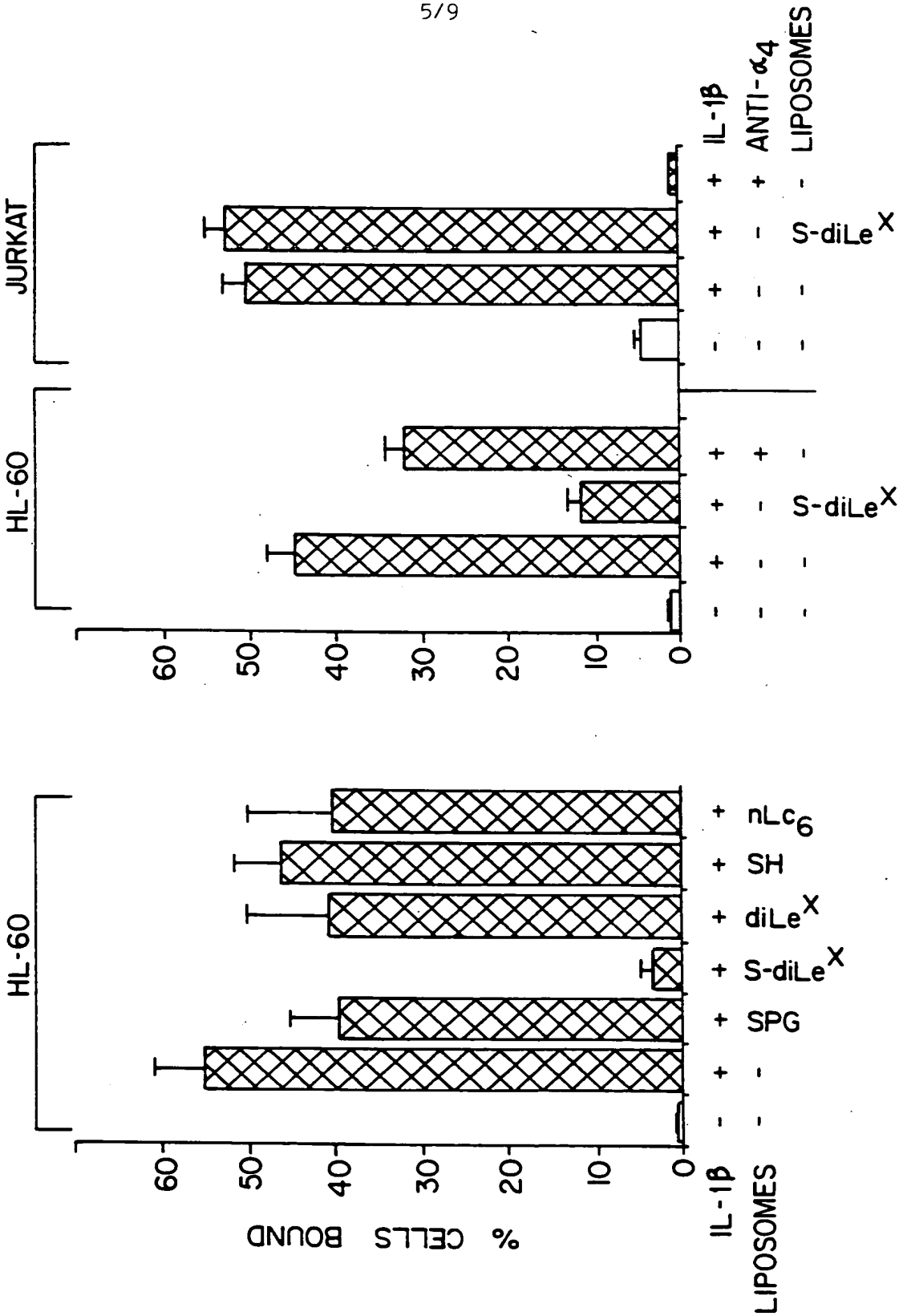

JURKAT
HL-60
HL-60
% CELLS BOUND
0
10
20
30
40
50
60
IL-1β
ANTI-α4
LIPOSOMES
S-diLe^X
S-diLe^X
nLc6
SH
diLe^X
S-diLe^X
SPG
IL-1β
LIPOSOMES

FIG. 5.

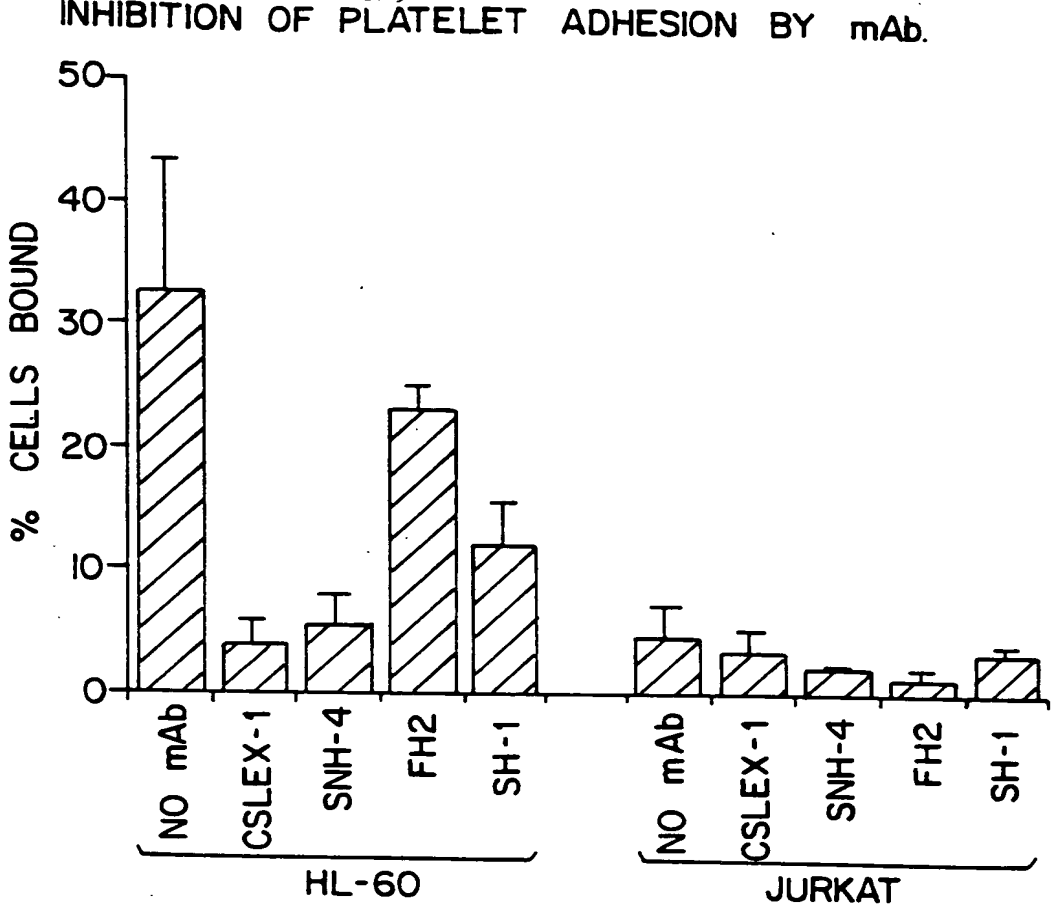

INHIBITION OF PLATELET ADHESION BY mAb.
% CELLS BOUND
50
40
30
20
10
0
NO mAb
CSLEX-1
SNH-4
FH2
SH-1
HL-60
JURKAT

FIG. 6.

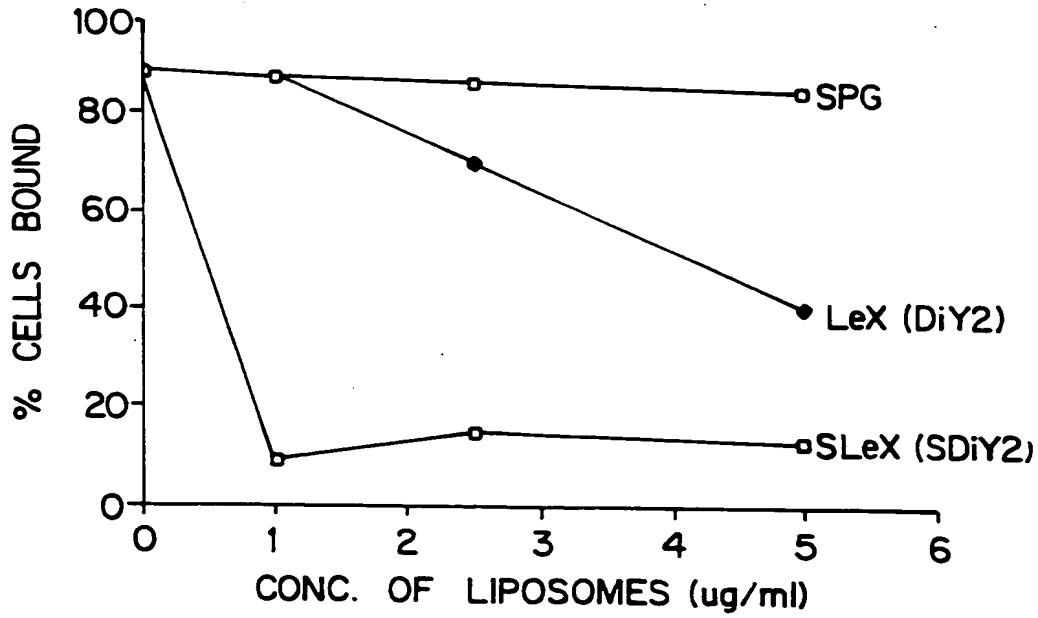

% CELLS BOUND
100
80
60
40
20
0
SPG
LeX (DiY2)
SLeX (SDiY2)
0
1
2
3
4
5
6
CONC. OF LIPOSOMES (ug/ml)

FIG. 7.

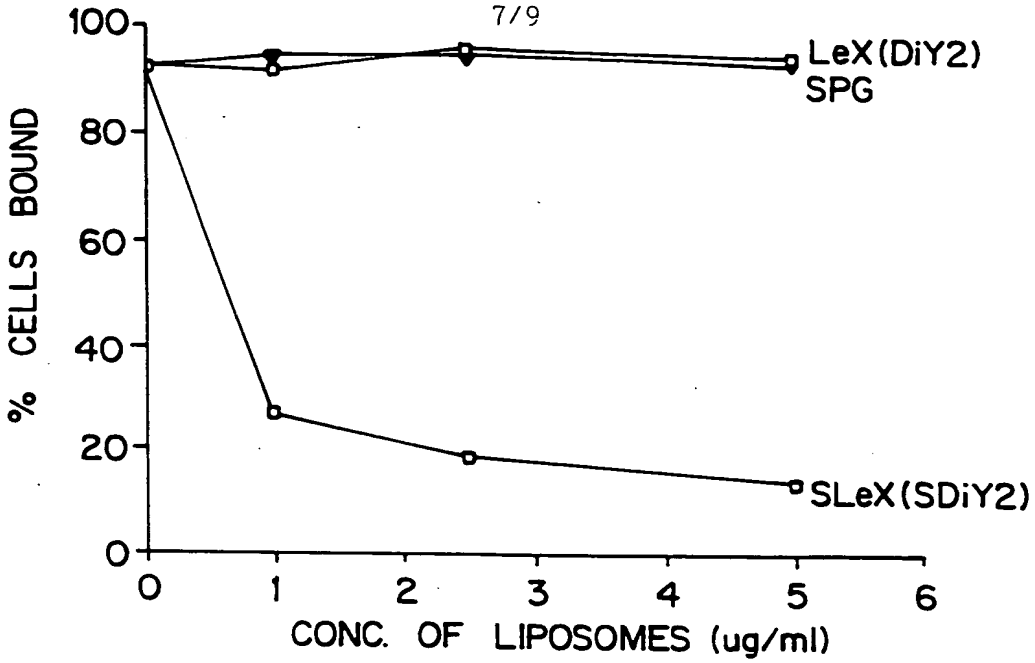
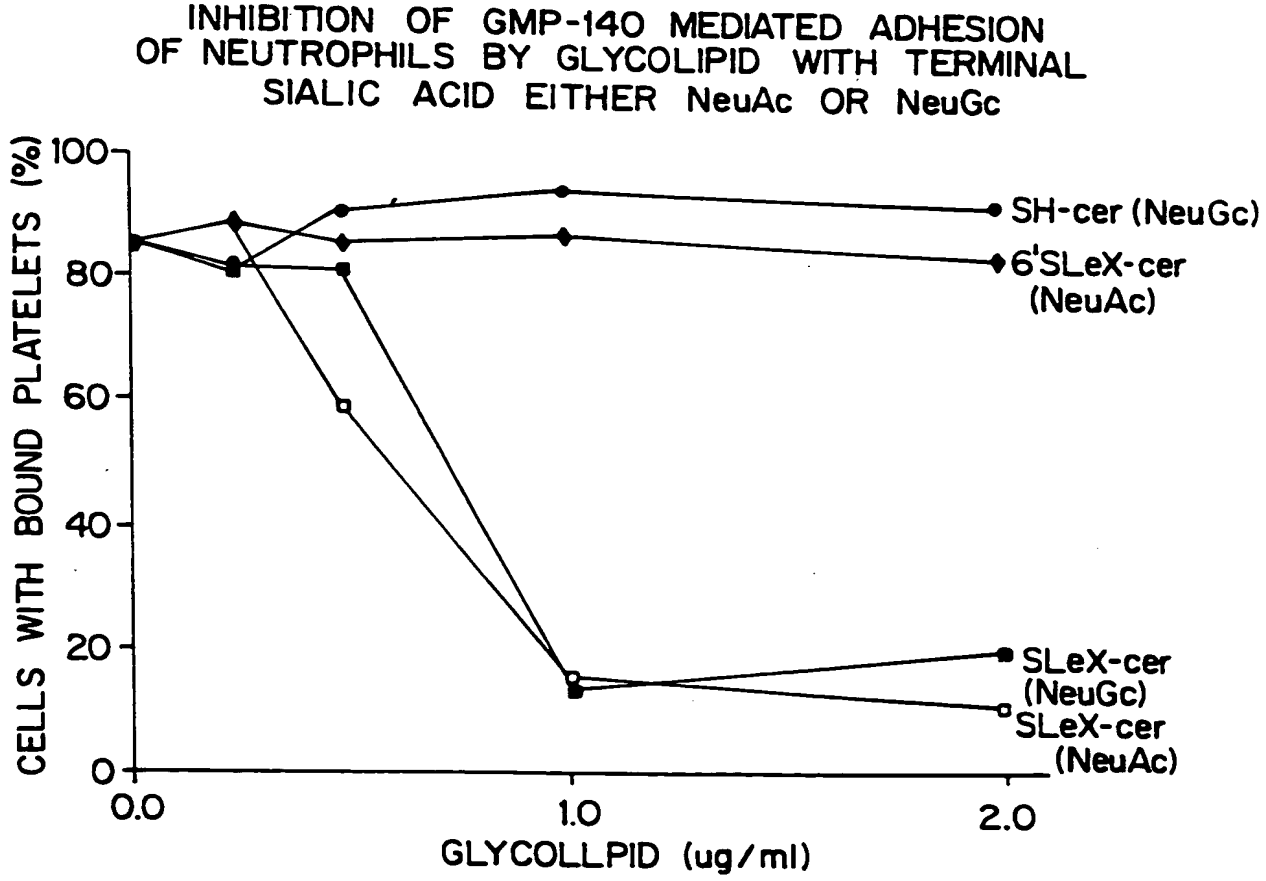


FIG. 8.



SUBSTITUTE SHEET

FIG. 9.

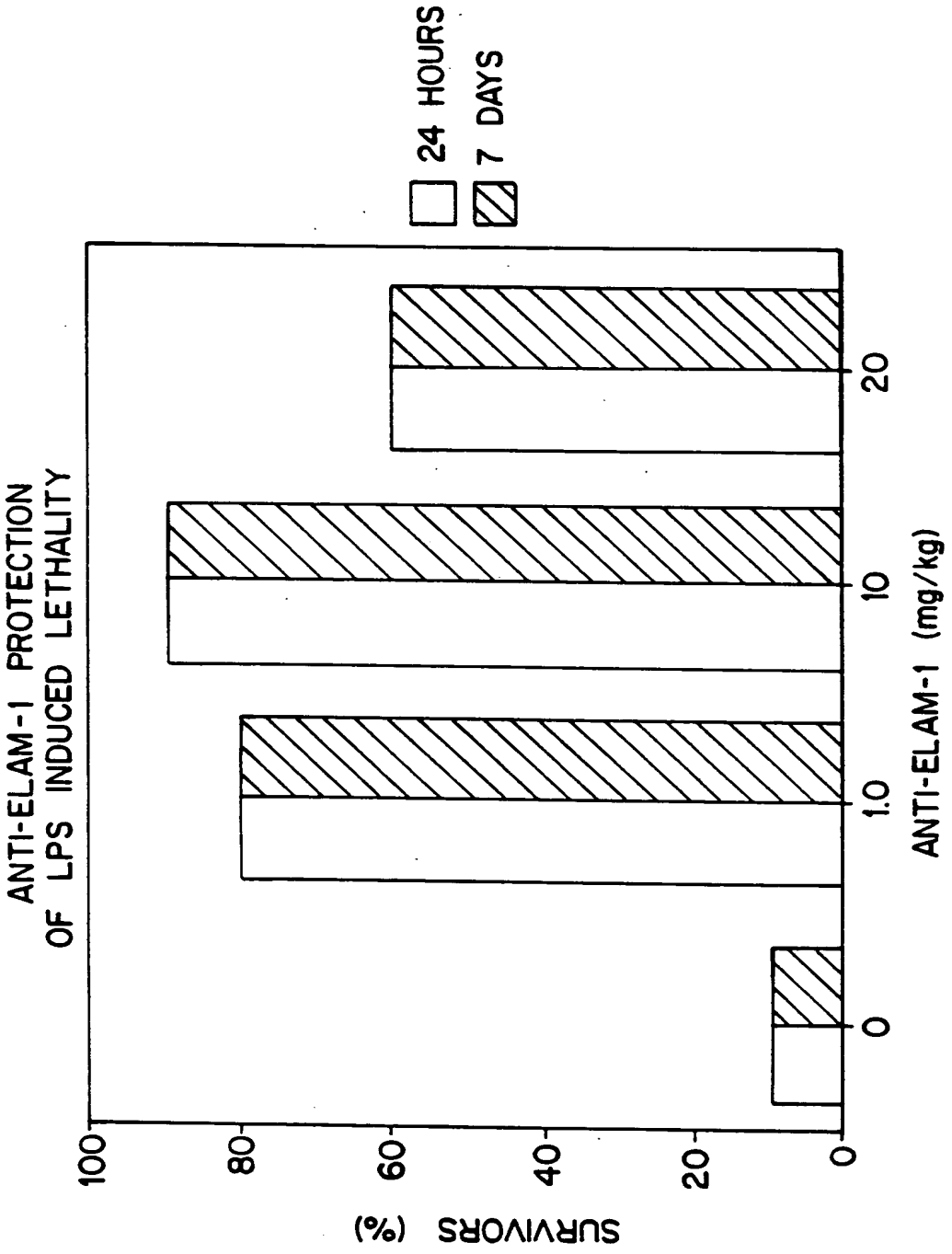

ANTI-ELAM-1 PROTECTION
OF LPS INDUCED LETHALITY
24 HOURS
7 DAYS
SURVIVORS (%)
100
80
60
40
20
0
0
1.0
10
20
ANTI-ELAM-1 (mg/kg)

FIG. 10.

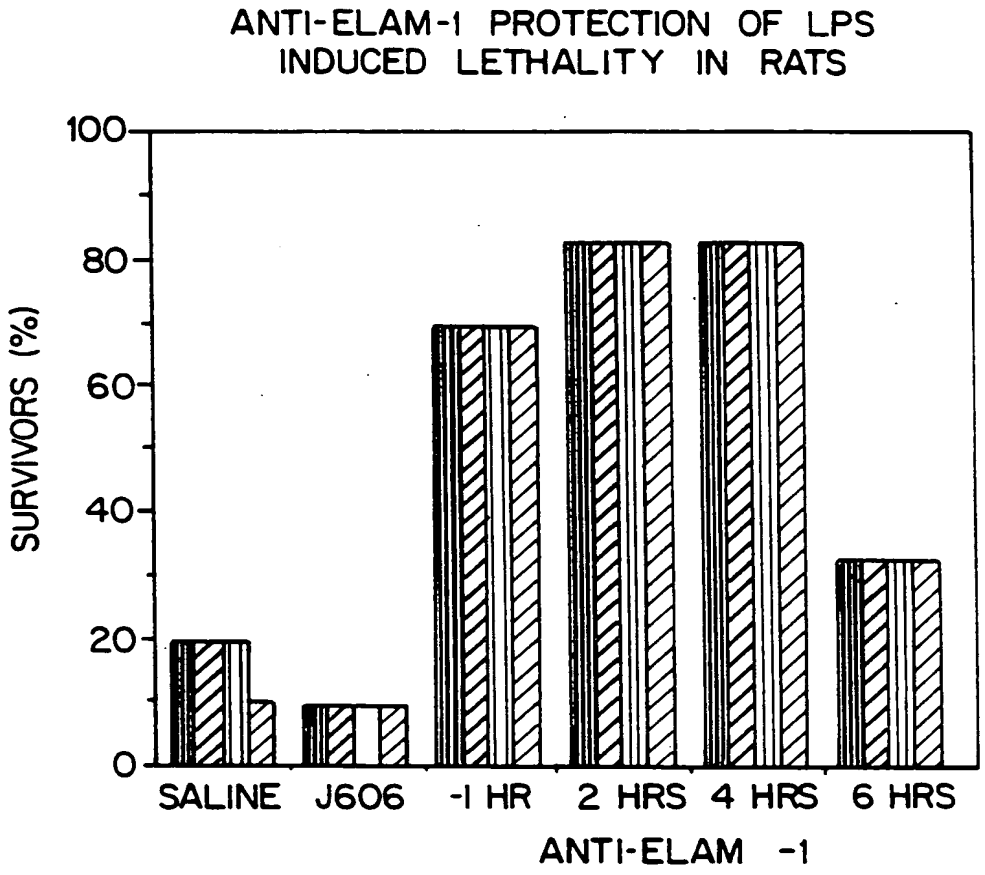

ANTI-ELAM-1 PROTECTION OF LPS
INDUCED LETHALITY IN RATS
SURVIVORS (%)
100
80
60
40
20
0
SALINE
J606
-1 HR
2 HRS
4 HRS
6 HRS
ANTI-ELAM -1

FIG. 11.